The Role of Pyruvate Dehydrogenase Kinase-4

in Post-Exercise Glycogen Recovery

Eric A.F. Herbst, BKIN

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Under the supervision of Sandra Jane Peters, PhD

Faculty of Applied Health Sciences
Brock University
St. Catharines, Ontario
L2S 3A1

"A few years ago, as the story goes, scientists at NASA developed an interest in the bumblebee. The lab folks reckoned that the little insect held some secrets of flight that may provide answers about operating in space. After all, they asked, how could such small wings produce efficient lift for a relatively large and hairy torso? And how could a round body and flight position that violated many principles of aerodynamics move so effectively through the air? Indeed, there was much to learn.

So scientists set about studying the bumblebee to discover its flying secrets. As scientists always do, they hypothesized about, scrutinized, examined, dissected, measured, timed, filmed, observed, compared, quantified, and debated the bumblebee. After weeks of study they came to one conclusion: Bumblebees are not capable of flight.

Fortunately, no one told the bumblebee. They go right on believing that flight is normal for them despite what the best minds in the scientific world know as fact.
We can learn a lot from the bumblebee. The single most critical piece of the puzzle is believing in yourself and your capacity to succeed. The bumblebee thinks it can fly. Actually, the thought of anything else never even crossed its mind. It just keeps flying"

- *Adapted from Friel, J., The Triathlete's Training Bible: Second Edition. Velopress, p.10, 2004.*

This thesis is dedicated to the bumblebees out there,

who never ask that people believe what they desire is possible,

only to have faith that if the possibility exists, they can do it

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This thesis would have not been possible without the help of an outstanding group of people, and therefore I would like to thank:

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Abstract

The pyruvate dehydrogenase (PDH) complex regulates the oxidation of carbohydrates in mammals. Decreased activation of PDH following exhaustive exercise may aid the resynthesis of glycogen through increased activity of PDH kinase-4 (PDK4), one of four kinases that decrease the activity of the PDH complex. The purpose of this study was to examine the role of PDK4 in post-exercise glycogen resynthesis. Wild-type (WT) and PDK4-knockout (PDK4-KO mice) were exercised to exhaustion and were sampled at rest (Rest), at exercise exhaustion (Exh), and after two-hours post-exercise (Rec). Differences in feeding post-exercise led to the addition of a PDK4-KO group, pair-fed (PF) with WT mice. Glycogen fully recovered in all Rec groups in muscle however remained low in the PF group in liver. Flux through PDH was elevated in PDK4-KO muscle with feeding and low in the PF group in both tissues. This suggests PDK4 may fine-tune flux through PDH during exercise recovery.

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List of Abbreviations

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
Ca^{2+}	calcium
CoA	coenzyme-A
DCA	dichloroacetate
E1	pyruvate dehydrogenase enzyme
E2	dihydrolipoamide acetyltransferase enzyme
E3	dihydrolipoamide dehydrogenase enzyme
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FADH	reduced flavin adenine dinucleotide
FFA	free fatty acid
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
GP	glycogen phosphorylase
GS	glycogen synthase
HK	hexokinase
kDa	kilodalton
K_i	inhibitory constant
K_m	Michaelis constant
LDH	lactate dehydrogenase
Mg^{2+}	magnesium
M_r	relative molecular weight
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
PDH	pyruvate dehydrogenase complex
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase
PCr	phosphocreatine
P_i	inorganic phosphate
PP-1	protein phosphatase-1
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
V_{max}	maximal enzyme velocity

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CHAPTER 1 - Introduction

In mammalian tissues, the regulation of carbohydrate oxidation is controlled by the pyruvate dehydrogenase (PDH) complex. This enzyme complex is situated on the inner mitochondrial membrane and controls the formation of acetyl-CoA from carbohydrate derivatives through the following reaction:

$$\text{pyruvate} + NAD^+ + CoA \text{ --------> acetyl-CoA} + NADH + H^+ + CO_2.$$

Increasing the activity state of PDH leads to increases in the rate of carbohydrate oxidation (110), and therefore the PDH complex is inevitably responsible for the fate of glucose once entered the tissue. Compared to the body's fat stores, glucose stored in glycogen offers a quickly mobilized and oxidizable substrate for energy provision, however as these stores are not as abundant, adequate control of their oxidation through the PDH complex is necessary to maintain euglycemia. Uniquely, the PDH complex has been shown to be able to tailor the flux of oxidation acutely, to quickly adapt in response to metabolic perturbations such as the onset of exercise, as well as chronically, to allow for sustained long-term inhibition with situations such as fasting. As the PDH complex plays a vital role in whole body glucose homeostasis, the understanding of this complex may also be important during the regulation of glycogen resynthesis following exercise. The flux through PDH has been previously shown to decrease during exercise recovery (52, 70), favouring increased recycling of three-carbon intermediates such as lactate and alanine for glycogen resynthesis. Although an exact mechanism for this has yet to be determined, growing evidence suggests that PDH activation (PDHa activity) is attenuated during recovery by PDK4 (38, 78, 80), one of four regulatory PDH kinases. Therefore,

the purpose of this thesis was to further elucidate the role of PDH kinase isoform 4 in regulating the PDH complex post-exercise.

CHAPTER 2 - Literature Review

Structure & Reactions of the PDH Complex

The structure of the mammalian PDH complex is comprised of three enzymes that are responsible for transforming pyruvate into acetyl-CoA. The dihydrolipoamide acetyltransferase enzyme (termed E2) makes up the 60-subunit inner pentagonal-dodecahedral core (reviewed in (111)). This E2 core establishes a framework from where a class of branching extremities extend that each contain a carboxyl-terminal catalytic domain, a binding domain for the pyruvate dehydrogenase enzyme (termed E1), and two further lipoyl domains (reviewed in (88)) (see Figure 1). Each of these divisions are connected through the use of linker hinge regions labeled H1, H2, and H3, in respect to their location when moving inward from the most distal region of the complex. Also extending from the E2 is a second class of branching limbs, linked through hinge regions, that work outward to connect a single lipoyl domain (L3) and the third enzyme, dihydrolipoamide dehydrogenase (E3), via an E3 binding protein (E3BP) (74, 116). The manner in which the E3-binding limb attaches to the complex is not well understood, however recent evidence suggests that the C-terminal inner domain of the E3 appendage may substitute that of the E1-bound limb at key locations around the enzyme (39). Considering this possibility, 12 of the 60 branching extremities of the complex would comprise the attachments for the 12 E3BPs and 24 E3 enzymes observed to be present in the complex (reviewed in (36)).

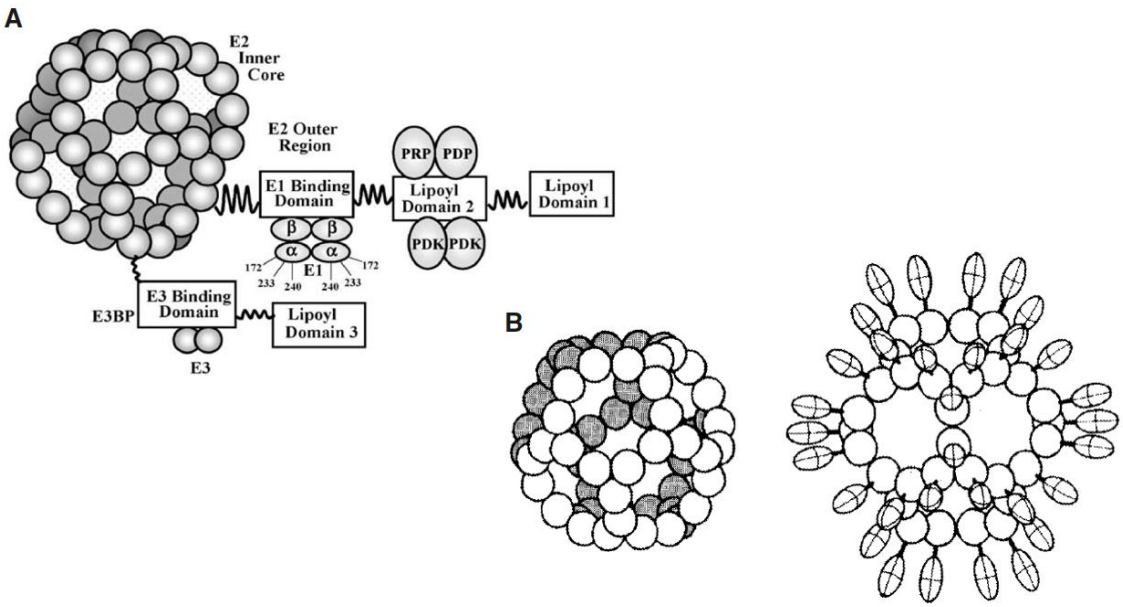

Figure 1 - The orientation and structure of A) the branching extremities off of the PDH E2, as laid out by Harris (36), and B) the inner E2 (left) and outer E1 and E3 regions (right), as represented by Reed (89).

The E1 enzyme is a 154 kDa, $\alpha 2\beta 2$, TPP-dependent heterotetramer (6) that binds to the complex via the E1 binding domain. The E1 catalyzes the initial two stages of the PDH reaction through decarboxylating pyruvate, releasing CO_2, and reductively acetylating the L1 lipoyl moiety (reviewed by Roche (93)). The E1 possesses the only non-equilibrium reaction involved with the PDH complex (see Figure 2) that in turn fully commits pyruvate to oxidation. Furthermore, the distal portion of the E1 binding domain may act as a lever for which the remaining extremity can operate as a biological crane to interact with its surrounding environment, potentially assisting in substrate/product shuttling between catalytic sites and lipoyl moieties (reviewed by Reed (88)). Following the E1 reactions, the 3,600 kDa E2 enzyme (39) transfers the acetyl group produced by E1 to free coenzyme-A (CoA) to form acetyl-CoA (reviewed by Behal (7)). At this point

the reduced lipoyl moiety becomes reoxidized via two reactions catalyzed by the 110 kDa FAD-dependent E3 homodimer (reviewed by Mattevi (69)), reducing NAD^+ to NADH.

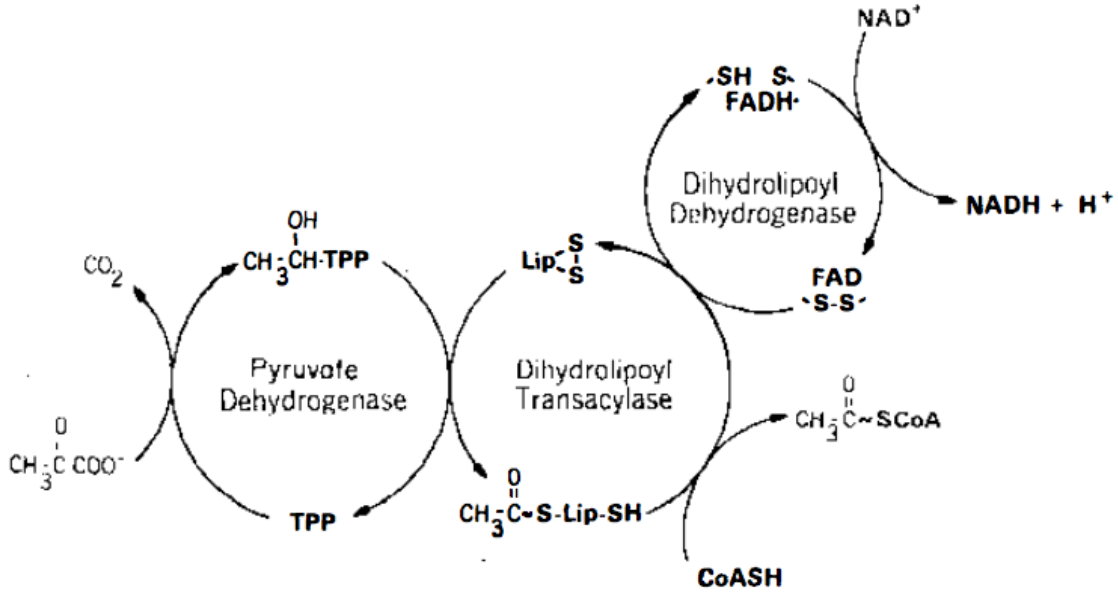

Figure 2 - The reactions of the PDH complex taken from Behal *et al*. (7). Take note that this diagram does not identify the near-equilibrium reactions of the dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) enzymes. Abbreviations include: thiamine pyrophosphate (TPP); lipoyl moiety (Lip); pyruvate (CH_3COCOO^-); flavin adenine dinucleotide in its oxidized (FAD) and reduced (FADH) forms; nicotinamide adenine dinucleotide in its oxidized (NAD^+) and reduced (NADH) forms; an acetyl group (CH_3CO); and coenzyme-A (CoA or CoASH).

Regulation of the PDH Complex

The PDH complex is regulated through a phosphorylation-dephosphorylation cycle by two unique classifications of isoenzymes (66). Four pyruvate dehydrogenase kinases (PDK1-4) and two pyruvate dehydrogenase phosphatases (PDP1-2) target the α-subunit of E1 (E1α) where three regulatory serine phosphorylation sites are situated (117). Phosphorylation of any one of these sites by any PDK will sufficiently inactivate E1 on that branch of the complex and interestingly, has been shown not to hinder subsequent phosphorylation of other residues (56). The rate of phosphorylation, however, is site specific, with site 1 (serine-264) phosphorylation occurring most rapidly, followed by that of site 2 (serine-271), and then site 3 (serine-203) (55). Site 1 also seems to receive phosphorylation most frequently, well over that observed on sites 2 and 3 (96). In contrast, dephosphorylation of these three sites by a PDP occurs most rapidly at site 2, followed by site 3, and then site 1 (55). This reactivation of the complex has been shown to be attenuated by phosphorylation of multiple sites in tandem (104), which in addition to observations *in vitro*, has been demonstrated most dramatically *in vivo* in starved and diabetic rats (97). The extent of phosphorylation, however, occurs relative to the concentrations of intramitochondrial effectors stimulating the kinases (9).

The Pyruvate Dehydrogenase Kinases

Each of the PDK isoforms (PDK1-4) are 45-47 kDa heterodimers comprised of a catalytic domain and a regulatory domain, where ATP and ADP bind to the catalytic domain, and pyruvate and the lipoyl group of L2 bind to the regulatory domain (101). Each of these isoforms interact through potassium-dependent association with the lipoyl domains of the complex (40) and demonstrates unique tissue expression and distinct kinetic properties in response to different cellular metabolites.

PDK1

PDK1 expression has been observed almost exclusively in the heart (9, 82), with only mild expression observed in soleus, red gastrocnemius (76), and other tissues (82). In humans, the PDK1 isoenzyme has been found to be 93% homologous with its rat counterpart, and in addition to its abundance in the heart, has exhibited expression to a lesser extent in skeletal muscle, liver, pancreas, brain, placenta, lung, and kidney (34). PDK1 is the only kinase to also phosphorylate site 3 of E1α (54, 56) and does so at a slightly slower rate than performed on site 1, but marginally faster than that of site 2 (54). Relative to the other kinases *in vitro*, PDK1 has exhibited a high specific activity and a strong insensitivity to the presence of ADP (9). Furthermore, PDK1 activity increases with a rise in the ratio of $NADH/NAD^+$, which is further augmented with the addition of acetyl-CoA (see Table 1).

PDK2

PDK2 is the most abundant PDK isoenzyme in mammals as it has been observed to be expressed ubiquitously in rodent (9) and human (34) tissues, with 93% homology between species. *In vitro*, PDK2 has a low specific activity (9) and incorporates much less phosphate into site 1 than the other isoenzymes, making dephosphorylation by the PDPs easier compared to when site 1 phosphorylation is performed by other kinases (54). In addition, this isoenzyme seems to display a preference towards site 1, and phosphorylates this site 19-fold faster than site 2 (54). Although PDK2 displays the greatest sensitivity towards binding of the non-metabolizable pyruvate analogue, dichloroacetate (DCA), the physiological relevance of the ability of DCA to inhibit any of the PDK isoenzymes occurs in the presence of ADP, where binding of DCA to the regulatory site lowers the inhibitory constant (Ki) of ADP so that both inhibitors may act synergistically to attenuate kinase activity (9, 84). It is worth noting that this effect may be increased with pyruvate, as pyruvate has been shown to be a more potent inhibitor of PDK2 than DCA (3). In contrast, PDK2 activity is stimulated in the presence of NADH only when accompanied by acetyl-CoA (see Table 1) (9). The unique sensitivity to both substrate-stimulation and product-inhibition by this isoenzyme makes PDK2 the predominant day-to-day regulator of PDH activity in most of the body's tissues.

PDK3

In 1995, PDK3 was isolated for the first time in human tissues (34). Comparison of amino acid sequencing showed a 67-68% homology with PDK1 and PDK2. In humans PDK3 is found almost exclusively in the heart and skeletal muscle (34), however, unlike the other PDK isoforms, rodent PDK3 distribution is not analogous with that found in humans and is contrarily expressed predominantly in the testes, with smaller expression in lung, kidney, spleen, brain, and heart (9). *In vivo* experiments with this isoenzyme are difficult to perform as PDK3 expression is low and detection requires an antibody with high-specific binding (114). PDK3 phosphorylates site 1 approximately 6-fold more rapidly than site 2 (54), and in regards to its relative kinetic parameters, PDK3 has presented specific activity two-fold higher than any other isoform, higher sensitivity to inhibition by ADP, and relatively low sensitivity to the presence of DCA (see Table 1) (9). Experiments with this isoenzyme have additionally shown that it does not react to changes in the ratio of NAD^+/NADH, and counterintuitively, PDK3 unexplainably displays decreased activity in the presence of acetyl-CoA (9).

	PDK1	PDK2	PDK3	PDK4
Specific Activity	650 ± 80	50 ± 5	1250 ± 200	400 ± 60
K_m for ATP (μM)	60 ± 5	10 ± 1	50 ± 5	65 ± 6
K_i for ADP (μM)	370 ± 20	120 ± 20	80 ± 10	100 ± 15
K_i for DCA (mM)	1.0 ± 0.2	0.20 ± 0.05	8.0 ± 1.0	0.5 ± 0.2
K_i for ADP in the presence of DCA (μM)	50 ± 10	35 ± 5	50 ± 10	200 ± 30
Kinase activity NAD^+/NADH (200:1)	460 ± 10	33 ± 2	1020 ± 80	220 ± 15
Kinase activity with NAD^+ /NADH (1:3)	540 ± 15	44 ± 5	1150 ± 90	410 ± 20
Kinase activity with NAD^+ /NADH (1:3) + 50μm acetyl-CoA	820 ± 30	105 ± 10	680 ± 50	340 ± 25

Table 1 - Kinetic parameters of the different pyruvate dehydrogenase kinases (PDKs). Abbreviations include: K_m (michaelis constant), K_i (inhibitory constant), DCA (dichloroacetate), and NAD^+ and NADH (nicotinamide adenine dinucleotide; oxidized and reduced forms respectively). Information adapted from Bowker-Kinley *et al*. (9).

PDK4

PDK4 was first cloned in 1996 during an investigation regarding genetic factors potentially related to type 2 diabetes in the native Pima Indians, a population with a high prevalence for the disease (95). In both rodents (9) and humans (95), PDK4 is abundantly expressed in heart and skeletal muscle, with lower quantities displayed in liver, lung, and kidney. PDK4 demonstrates a high specific activity (9) and shows no strong preference for site-specific activity, as site 1 phosphorylation by this isoenzyme occurs only 2.5-fold more rapidly than site 2 (54). Compared to PDK2, PDK4 is unique in that it has double the K_i towards DCA as well as a five-fold higher K_i towards ADP in the presence of DCA than any other kinase (see Table 1) (9). This property makes PDK4 distinctly unresponsive to local changes in pyruvate concentrations while allowing attenuation of PDH activity when necessary, regardless of the rate of pyruvate formation. The activity state of PDK4 occurs independent of acetyl-CoA concentrations and is stimulated solely through increases in the $NADH/NAD^+$ ratio (9). PDK4 has also been shown to be responsive to increased lipid availability, such as with fasting (49) or a chronic high-fat diet (46, 48), reducing the flux of pyruvate to acetyl-CoA so it may potentially be used for gluconeogenesis.

The Pyruvate Dehydrogenase Phosphatases

The 150 kDa PDP isoforms are made up of a 53 kDa catalytic subunit and a 96 kDa regulatory subunit (58), which are together responsible for dephosphorylating the serine residues on E1α. Until the late 1990's it was believed that a single PDP isoform performed this in all tissues, however to date two isoenzymes have been identified to exist in both rodents (44) and humans (86).

PDP1

The PDP1 isoform is expressed abundantly in the heart, brain, and testes, with minor expression in other tissues (44, 45). Additionally, PDP1 is the dominant phosphatase present in skeletal muscle (59, 61) and for this reason the sensitivity of PDP1 to local changes in Ca^{2+} concentrations is essential for proper activation of PDH (45). Previous research has identified Ca^{2+} as a dominant Mg^{2+}-dependent activator of PDP activity (23, 58, 100, 105, 115) as well as an enhancer of PDP binding to L2 of the PDH complex (15, 106). Further demonstrating the responsiveness of this isoform to Ca^{2+}, incubating PDP1 in elevated Ca^{2+} concentrations increases the maximum enzymatic velocity (V_{max}) and simultaneously decreases the K_m for Mg^{2+} (44), promoting enhanced activity.

PDP2

Observed variation in the kinetic parameters of PDP between tissues led to the investigation, discovery, and cloning of the PDP2 isoenzyme in rodents (44). PDP2 has been observed to be expressed in 3T3-L1 adipocytes, liver, heart, brain, and kidney, with lesser expression in spleen, lung (44, 45), and skeletal muscle (59, 61). PDP2 has a

peptide sequence that shares 55% homology with its PDP1 counterpart and has 90% conservation in regions considered structurally vital to PDP function (44). *In vitro*, PDP2 has demonstrated a relative insensitivity towards physiological concentrations of Ca^{2+}, which may be an important trait of this isoenzyme in PDP2 expressing tissues. It has been shown *in vitro*, however, that in the presence of Ca^{2+} accompanied with high concentrations of Mg^{2+}, PDP2 can elicit a higher maximal activity than PDP1 (44). Also interesting, spermine, which has been shown to stimulate mitochondrial Ca^{2+} uptake (63), elicits a 5-fold increase in the sensitivity of PDP2 to Mg^{2+} with a concomitant one-third decrease in its V_{max} (44), suggesting that PDP2 activity may not be entirely independent of intracellular changes in Ca^{2+} activity.

Insulin has also been demonstrated to stimulate PDP2 activity (14, 57, 65, 71, 83), and although evidence suggests a direct role through the involvement of protein kinase C-delta (14), a downstream target of insulin involved in multiple signaling pathways (64), mutagenesis studies disrupting the function of the tyrosine kinase insulin receptor have witnessed no impairments of the effect of insulin on PDP stimulation (32), leaving the exact mechanism of insulin action on PDP2 unknown.

Regulation of Carbohydrate Oxidation During Exercise

During the early stages of prolonged exercise, muscle PDHa activity rises and begins to decline after a few hours of activity as exhaustion approaches, at which point muscle gradually relies more heavily on the oxidation of fat, sparing glycogen (18, 50, 70, 108). This effect is also seen with endurance training and is further characterized by a lower activity of PDHa for a given workload (60). It is currently thought that these changes are mediated by alterations in the expression and stimulation of the regulatory isoenzymes of PDH. Although there is currently little evidence to suggest changes in PDP activity occur throughout an exercise bout, it has been demonstrated that PDK activity increases with observed decreases in PDHa activity (109). It is not entirely clear which of the PDK isoforms are responsible for attenuation of PDH during exercise, however PDK2 seems to be most probable, as it is the only kinase to increase expression following endurance training (62). Examining the specific role of PDK2 during exercise has proven difficult, however, due to the important role this kinase plays in the habitual regulation of the PDH complex. PDK2-knockout (KO) mice, which are devoid of PDK2 expression, experience rapid compensation from the less abundant PDK1 during development (25), potently inhibiting the PDH complex during muscle contraction (24). Alternatively, PDK4 has also been implicated in PDH regulation during exercise, as studies have documented increased PDK4 transcription at exercise cessation (38, 70, 79) and further studies examining the role of PDK4 during muscle contraction in PDK4-KO mice have observed impaired PDH regulation and unrestricted PDHa activation at higher intensities of muscle contraction (Herbst *et al*., unpublished data), providing support for the involvement of PDK4 during exercise.

Glycogen Metabolism

At rest, liver glycogen stores supply the blood with adequate glucose to maintain plasma glucose concentrations, however in situations that tax these stores, such as with fasting or prolonged exercise (30), tissues such as the heart and skeletal muscle must rely on their own glycogen stores. Although the PDH complex ultimately determines whether the majority of carbohydrates will either be oxidized or used for gluconeogenic purposes, other metabolic factors exist upstream of glucose oxidation that regulate the formation and breakdown of glycogen.

Glucose that enters the cell is converted to glucose-6-phosphate (G-6-P) by hexokinase (HK) and may then either enter glycolysis or be converted to glycogen (glycogenesis). G-6-P committed to glycogenesis becomes converted to glucose-1-phosphate (G-1-P) by phosphoglucomutase, and then to UDP-glucose via UDP-glucose pyrophosphorylase, where it can be used as a substrate for glycogen synthesis (1) (see Figure 3). At the centre and beginning of each glycogen molecule lies a 37-38 kDa self-glucosylating protein known as glycogenin. Glycogenin is located bound to the actin cytoskeleton (5) and uses UDP-glucose to perform the initial steps of glucosylation, adding 7-11 glucose molecules to itself in order to form an extending linear polysaccharide chain from which further glycogen growth can occur (2).

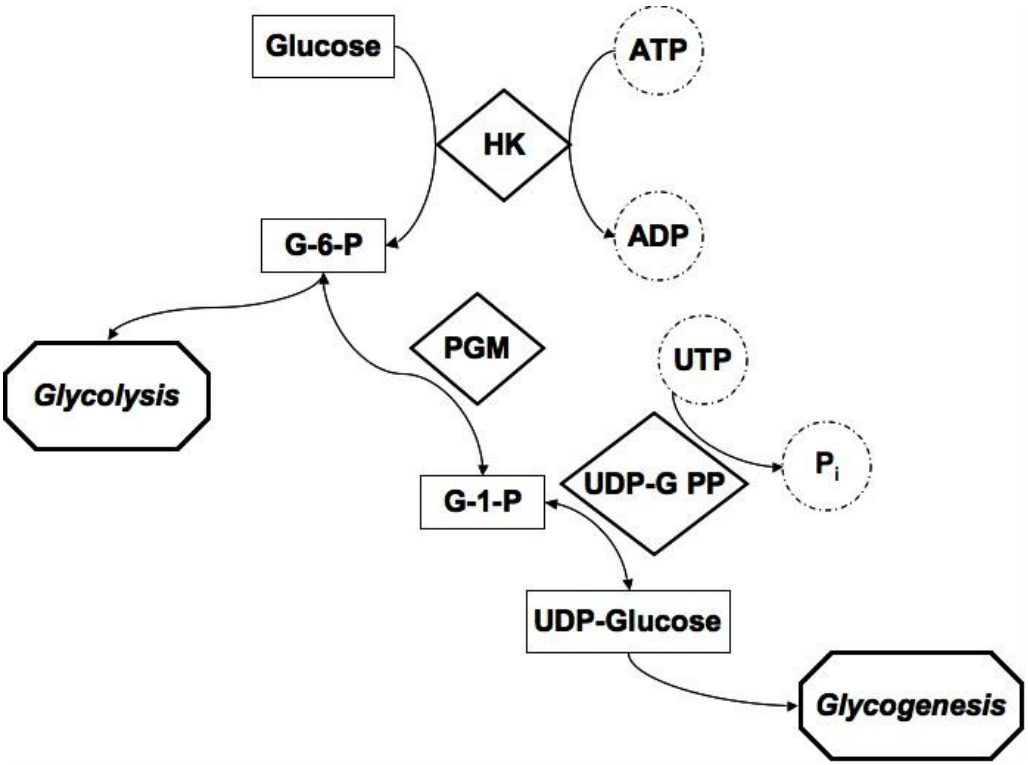

Figure 3 – The path of glycogenesis. Glucose is converted to glucose-6-phosphate (G-6-P) by hexokinase (HK) then to glucose-1-phosphate (G-1-P) by phosphoglucomutase (PGM). Finally, through the breakdown of uridine triphosphate (UTP), G-1-P is converted to UDP-glucose by UDP-glucose pyrophosphorylase (UDP-G PP) for use in glycogen synthesis. Glycolysis represents an early alternative, yet an almost inevitable fate of glucose.

Glycogen Synthase

Glycogenin has limits to the extent it can self-glucosylate and cells must rely on additional enzymes to further promote glycogen synthesis. Glycogen synthase (GS) also uses UDP-glucose as a substrate and, with the assistance of a branching enzyme, adds diverging limbs to the polysaccharide chain bound to glycogenin to further expand the size of the glycogen molecule (see Figure 4). Two products are likely to form from this: a smaller 400 kDa intermediate product termed proglycogen, or an often final 10,000 kDa product called macroglycogen (67).

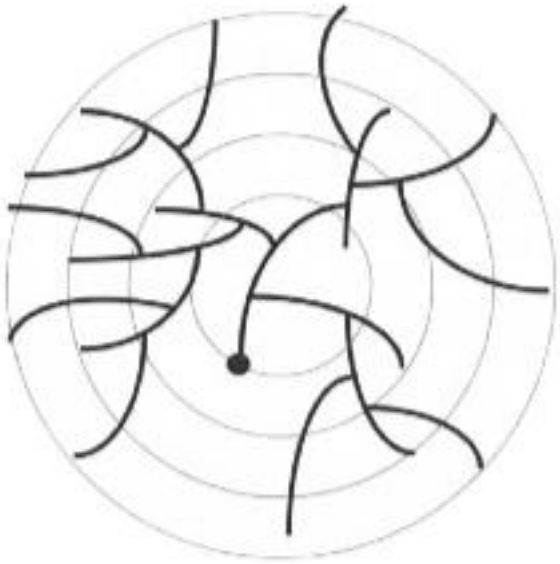

Figure 4 – The Structure of a glycogen molecule. The filled circle in the centre represents the glycogenin protein from which a polysaccharide chain extends. Extending from this chain are further tiers of glucosyl branches synthesized by glycogen synthase. Figure adopted from Roche et al. (91).

In both humans and rodents, GS is expressed as separate isoforms in liver (73) and skeletal muscle (12), with ~70% sequence identity with one another, suggesting that the regulation of GS likely differs between tissues. Furthermore, multiple kinases and phosphatases have also demonstrated the capability of phosphorylating and dephosphorylating GS to different degrees, in a tissue specific manner, to either enhance or reduce glycogen synthesis (10, 13, 28, 29, 72, 90-92), respectively. None of these regulatory enzymes are associated with GS exclusively, however, and each also plays additional roles in alternative metabolic signaling cascades, making their application *in vivo* more difficult to study. Therefore, although understanding the regulation of GS activity is continually advancing, the specifics regarding the relative importance of the various kinases and phosphatases in the covalent regulation of GS is still not entirely clear.

GS activity is also subject to allosteric modification and is stimulated by the presence of G-6-P regardless of the state of phosphorylation, promoting enhanced glycogen synthesis with increases in glucose uptake (81). During recovery from exercise, attenuation of PDHa activity would theoretically play a role in further promoting glycogen synthesis, as reduced oxidation of carbohydrates would allow for greater recycling of carbohydrate derivatives towards glycogen synthesis.

Recovery from Exercise

Glycogen concentrations in muscle (75), liver (4), and heart (19) are markedly decreased following exhaustive exercise, with rapid resynthesis of glycogen occurring post-exercise. Studies examining glycogen recovery following exhaustive exercise have shown that muscle glycogen resynthesis takes preference over liver replenishment, as rats fasted following exhaustive exercise demonstrate full recovery of muscle glycogen within 24-hours despite the absence of dietary glucose intake (27). Furthermore, glycogen stores in skeletal muscle and heart have shown the ability to supercompensate following exercise, however where skeletal muscle glycogen has been shown to remain elevated for a few days following exercise (8, 31), heart glycogen recedes to baseline levels within 24-hours following exhaustion (19, 98, 99). Glycogen repletion in either of these tissues is reliant on glucose provided to the circulation through diet, exogenously through the breakdown of remaining liver glycogen, as well as through gluconeogenesis from lactate, alanine, and glycerol (94).

Decreases in the activity of PDHa have been observed following exhaustive exercise in humans (52, 70), which hypothetically would allow for greater shift of pyruvate to lactate and alanine, leading to a greater recycling of these three-carbon substrates and improved glycogen resynthesis. Kimber *et al.* (52) examined the contributions of fat oxidation during recovery from glycogen depleting exercise and demonstrated an increased reliance on free fatty acids (FFA) along with decreased PDHa activity with glycogen recovery, supporting a role for PDHa attenuation post-exercise. PDK2 activity is potently stimulated by high acetyl-CoA concentrations, and although no mechanism was suggested for the observed inhibition of PDH in this study, the

concentrations of acetyl-CoA observed were low during recovery, suggesting that another kinase may be responsible for PDHa attenuation. Increased FFA availability has been shown to stimulate PDK4 expression (16), and although no changes in PDK4 protein content have been observed, increases in PDK4 transcription (38, 79, 80) and mRNA content (79, 80) have been observed following exercise in both human and rodent skeletal muscle, suggesting that PDK4 may be responsible for reducing PDHa activity following exercise-induced exhaustion. Depletion of glycogen stores post-exercise has also been suggested to share a link with increased PDK4 transcription (78) and protein expression (51), however studies that reduced glycogen content prior to exercise in humans also reduced the availability of carbohydrates and promoted increased reliance on FFA as a consequence. Increased FFA availability alone has been shown to stimulate peroxisome proliferator-activated receptor alpha (PPARα) in the rodent model (17), which has been demonstrated to increase the transcription and protein expression of PDK4 (112). Therefore, although PDK4 may be the dominant mechanism for the inhibition of PDHa activity post-exercise, isolating the role of PDK4 during exercise recovery without stimulating PDK4 activity by other means has proven difficult. As such, the extent of PDK4 involvement during post-exercise glycogen resynthesis is still unclear.

Justification for the PDK4-KO Model

In addition to the potential involvement of PDK4 during exercise and recovery, the expression of this isoenzyme has been shown to increase with changes in the abundance of carbohydrates, such as with diabetes (113, 114), long-term fasting (49, 76, 103, 113, 114), and high-fat feeding (48, 77). The PDK4-KO model has been previously implemented to investigate the extent of PDK4 involvement under these circumstances (46, 48, 49) and would also provide an experimental approach to examine the role of PDK4 in exercise recovery without directly manipulating the availability of carbohydrates. PDK4-KO mice have been previously characterized and show increased resistance to the effects of high-fat feeding (46, 48), increased glucose tolerance, and an impaired ability to adapt to long-term fasting (49). In the resting fed state, PDK4-KO mice have higher PDHa activity in diaphragm and demonstrate compensation through increased expression of PDK2 in heart and kidney due to the absence of PDK4 (49). As no compensation by PDK2 has been shown to occur in liver or skeletal muscle of PDK4-KO mice (36-38), examining the repletion of glycogen following exercise in PDK4-KO mice may provide insight into the potential role of PDK4 during glycogen resynthesis.

Statement of the Problem

To date, there is limited evidence to support that PDHa activity decreases during recovery following glycogen-depleting exercise. Furthermore, studies attempting to elucidate a mechanism by which PDHa activity is reduced have only measured changes in the transcriptional expression and mRNA content of PDK4, a regulatory kinase that experiences increased transcription with increases in FFA availability.

Purpose

Therefore, the purpose of this study was to investigate a possible role for PDK4 in post-exercise glycogen recovery through examining the differences in the resynthesis of glycogen-depleted hindlimb and liver tissue of wild-type (WT) and PDK4-KO mice.

Hypothesis

It was hypothesized that PDK4-KO mice would experience impairments in their ability to resynthesize muscle glycogen following exhaustive exercise with only minor impairments in the resynthesis of liver glycogen.

CHAPTER 3 – The following is a manuscript that will be submitted to the Journal of Physiology.

The Role of Pyruvate Dehydrogenase Kinase-4 in Post-Exercise Glycogen Recovery

Herbst[1], Eric A.F., Rebecca E.K. MacPherson[1], Paul J. LeBlanc[1], Brian D. Roy[1], Nam Ho Jeoung[2], Robert A. Harris[2], and Sandra J. Peters[1]

[1]Centre for Muscle Metabolism and Biophysics, Faculty of Applied Health Sciences, Brock University, ON, CANADA, N1G 2W1; and [2]School of Medicine, Indiana University, Indianapolis, IN, 46202-5114

* Master of Science candidate; participated in study design with co-authors, executed study and biochemical analysis (excluding tissue genotyping), including development of assays, and wrote manuscript.

Introduction

Muscle glycogen resynthesis after exercise has received much focus since Bergtröm and Hultman first identified enhanced glycogen synthesis following glycogen-depleting exercise (8). Restoration of muscle glycogen is dependent on glucose supplied exogenously either through diet or gluconeogenesis. Although numerous precursors exist for hepatic gluconeogenesis, lactate and alanine represent the common products of muscle carbohydrate breakdown that are recycled through the liver for further glycogen synthesis (20, 22, 94). The mechanism for promoting the formation of these products post-exercise, however, is not fully understood. Decreased activity of the pyruvate dehydrogenase (PDH) complex may play a role, as the PDH complex is responsible for regulating the oxidation of carbohydrates in all mammalian tissues. PDH is inactivated and activated by four PDH kinases (PDK1-4) and two PDH phosphatases (PDP1-2), respectively, where PDK2 and PDP1 serve as the dominant regulators of PDH in resting skeletal muscle, and PDK2 and PDP2 are the principle regulators of liver PDH.

A decrease in PDH activation (PDHa activity) in skeletal muscle during recovery would decrease carbohydrate oxidation, increase the reliance on fat oxidation, and potentially promote an increased shift of pyruvate to lactate and alanine in muscle. This would increase the availability of gluconeogenic substrates for the liver, promoting enhanced glucose output for glycogen synthesis in depleted tissues (26). Kimber *et al*. (52) examined the contribution of fatty acids over an 18-hour period following prolonged glycogen-depleting exercise in human skeletal muscle and observed decreased PDHa activity along with a higher reliance on fat oxidation during glycogen recovery. Furthermore, Mourtzakis *et al.* (70) also examined changes in muscle PDHa activity

following an exhaustive exercise bout in humans and observed decreased PDHa activity along with increases in PDK4 mRNA content in the following hours of recovery, suggesting PDK4 stimulation may be a mechanism that promotes decreased carbohydrate oxidation through reducing PDHa activity. Multiple studies have additionally demonstrated increased PDK4 transcription and mRNA production during exercise recovery (38, 70, 78, 79), supporting that changes in PDK4 activity may reduce flux through PDH post-exercise. This approach is problematic, however, as elevated fatty acid availability, such as seen following exercise (52, 70), would be expected to stimulate PDK4 expression through peroxisome proliferator-activated receptor alpha (PPARα) (112), stimulating greater expression of PDK4 regardless of any need for PDK4 during recovery. Furthermore, PDK4 protein content has not been shown to change as a result of exercise, as no changes in PDK4 protein have been observed following 8-weeks of aerobic training in man (62). Therefore, although PDK4 may play a role in attenuating the activation of PDH to favour glycogen resynthesis, no evidence exists in the present literature to support this.

As the activity of the individual PDK isoforms cannot be isolated, determining the metabolic contributions of each isoform to PDH regulation is difficult. Therefore, the present study took a loss of function approach to examine the role of PDK4 in the regulation of PDH during recovery from glycogen-depleting exercise in skeletal muscle and liver of wild-type (WT) and PDK4-knockout (PDK4-KO) mice. We hypothesized that PDK4-KO mice would be less able to reduce the activity of PDHa in skeletal muscle during recovery and that glycogen resynthesis would be impaired due to loss of carbohydrates to oxidation through the PDH complex. As PDK4 is expressed to a greater

extent in skeletal muscle (9), it was expected that this effect would be more pronounced during resynthesis in muscle than in the liver.

Methods

Animals

Male PDK4-KO mice were generated from C57BL/6J mice by methods previously described (49) and were generously donated by Dr. Robert Harris (Indiana University School of Medicine). Male WT C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, QC) to serve as paired controls. All mice were housed on a 12-hour:12-hour reverse light:dark cycle in a controlled environment in the Brock University Animal Care Facility for at least 1-week prior to being used and had *ad libitum* access to mouse chow (5015, LabDiet, Aberfoyle, On) and water before the experiment and during exercise recovery. This study was approved by the Brock University Animal Care Committee and conforms to the standards of the Canadian Council for Animal Care.

Experimental Protocol

Mice were provided a running wheel 1-week prior to the day of the experiment to allow for familiarization with running. Each genotype was paired and separated into three groups consisting of a resting condition, and two exercise conditions. Exercising mice were brought to a dark room and placed on a treadmill (EXER 3/6, Columbus Instruments International, Columbus, Ohio) for 5-minutes to allow for familiarization with the treadmill environment. The exercise protocol used was adapted from a protocol previously shown to deplete muscle glycogen stores (75). Mice began running on a 20^{o} incline at an initial speed of 12-metres/minute with speed increases of 1m/min after 2-minutes, 5-minutes, 10-minutes, and every additional 10-minutes of exercise until

exhaustion. Exhaustion was determined by an inability to remain halfway up the treadmill before falling off the moving belt four times consecutively despite being poked and blown on. Resting (Rest) mice (n=6) received no special intervention before surgery and exercising mice were killed either at exercise exhaustion (Exh) (n=8) or after 2-hours of recovery (Rec) (n=8), with post-exercise food and water intake recorded. As differences in post-exercise nutrition between WT and PDK4-KO mice were observed during the 2-hour recovery period, a group of pair-fed (PF) PDK4-KO mice (n=8) was included for analysis with food rations weighed out to match average WT food intake. Average time from exercise exhaustion to completion of the surgery in Exh was approximately 15-min. All mice were anesthetized by an intraperitoneal injection of diluted sodium pentobarbital (6mg/100mg of body weight) and blood glucose (Freestyle, Abbott, Illinois, USA) and lactate (Prolactate Test Meter, Arkray, Japan) concentrations were sampled from the heart using hand-held devices. Whole blood samples for glucose and lactate analysis were taken through intracardiac puncture under anesthesia prior to euthanasia. Hindlimb muscle and liver were immediately harvested and instantly freeze-clamped for further analysis.

Western Blotting

Muscle tissue homogenization for WT and PDK4-KO mice at each time-point was performed on 10-15mg of tissue in a glass-on-glass potter homogenizer on ice in buffer containing 250mM sucrose, 100mM KCl, 5mM EDTA, with added protease (11836170001, Roach, QC) and phosphatase (04906845001, Roach, QC) inhibitors. Samples were diluted to a final concentration of 1.0μg/μL and loaded at 10μg

protein/lane. Proteins were separated by SDS-PAGE using a 10% separating gel for PDK2 and a 12% running gel for PDP1 before being transferred to polyvinylidene difluoride membrane. Membranes were incubated as previously described in 5% skim milk-TBST containing antibodies raised against PDK2 (25) (AP 7039b, Abgent), PDP1 (61) (MC-049, Kamiya Biomedical Company), and actin (612656, BD Transduction Laboratories), and again in solution containing secondary antibodies raised against the primary antibody (24, 61). All proteins were detected and quantified as previously described (25) before being normalized to actin as a loading control.

Glycogen and Metabolite Concentrations

Hindlimb muscle and liver were lyophilized, dissected free of connective tissue, and aliquoted for analysis of glycogen and metabolite concentrations. Glycogen aliquots were acidified in 2N HCl, heated at 100^{o}C for 2-hours, rehydrated, and neutralized in 2N NaOH. Metabolite aliquots were extracted in 0.5M $HClO_4$ and neutralized with 2.3M $KHCO_3$. The concentrations of glycogen, glycogen precursors (glucose and G-6-P), and gluconeogenic precursors (lactate and alanine) were analyzed in triplicate using fluorometric techniques as previously described by Harris et al. (37) and modified by Green et al. (33).

PDHa Activity

Wet muscle and liver tissue were used for the determination of PDHa activity as previously described (21, 85). Tissue was homogenized in buffer (pH 7.8) containing 200mM sucrose, 50mM KCl, 5mM $MgCl_2$, 5mM EGTA, 50mM Tris HCl, 50mM NaF,

5mM DCA, and 0.1% Triton X-100. Samples were freeze-thawed and assayed for PDHa activity in assay buffer (pH 7.8) containing 14.4mM Tris base, 0.72mM EDTA, 1.44mM $MgCl_2$, 1.1mM CoA, 1.1mM TPP, and 3.2mM NAD. The reaction was initiated with 1.4mM pyruvate, sampled at 1-, 2-, and 3-minutes, and placed in 0.5M $HClO_4$ to stop the reaction before being neutralized in 2.2M $KHCO_3$ and analyzed for acetyl-CoA concentrations.

Statistics

A Student's t-test was used to analyze differences between genotypes in time to exhaustion, post-exercise nutritional intake, and protein content. A two-way analysis of variance (ANOVA) (genotype x condition (Rest, Exh, Rec)) was used to examine differences among conditions between WT and PDK4-KO mice in glycogen content, metabolite concentrations, and PDHa activity. Because PF mice were only sampled at Rec, a one-way ANOVA was used to examine differences between groups (WT, PDK4-KO, PF) at Rec for glycogen content, metabolite concentrations, and PDHa activity. A Tukey's post-hoc test was performed if significance was detected ($p<0.05$). Due to low concentrations of muscle lactate and G-6-P in the PF group, some samples were not detectable and these groups did not meet the assumptions of normality and equal variances. Therefore a Kruskal-Wallis ANOVA on ranks with a Dunn post-hoc test was used to examine differences in muscle lactate and G-6-P concentrations at Rec between WT, PDK4-KO, and PF mice. All values are presented as mean ± S.E.

Results

Exercise and caloric intake during recovery

Absence of PDK4 had no effect on running time to exhaustion (p=0.898) (Figure 5). In the two-hour period following recovery, a 2-fold difference in food consumption was observed in PDK4-KO mice (p=0.005) (Table 2).

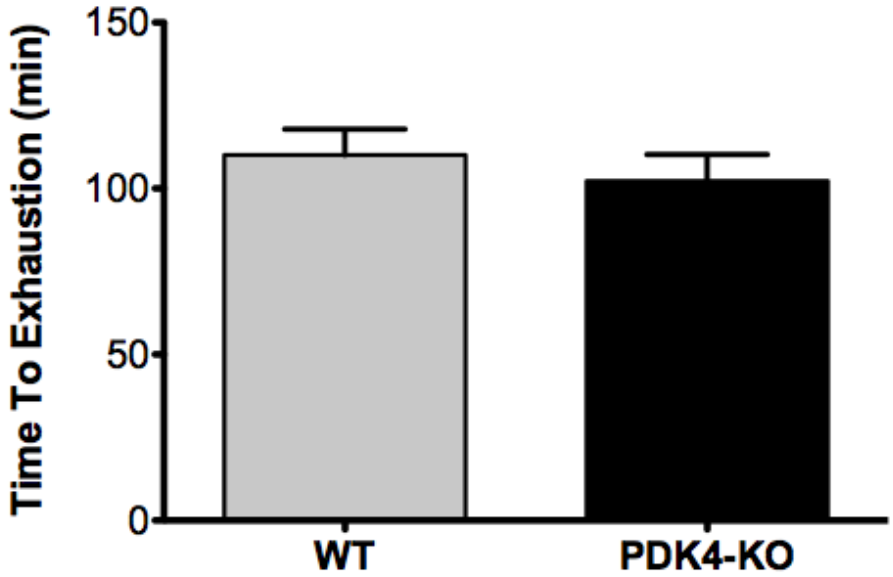

Figure 5 – Running time to exhaustion in wild-type (WT) and PDK4 knockout (PDK4-KO) mice (n = 16). Values are expressed as mean ± S.E.

	H_2O (mL)	Food (g)	Total kcal	Carb (kcal)	Fat (kcal)	Pro (kcal)
WT	2.44 ± 0.36	0.55 ± 0.09	2.57 ± 0.41	0.30 ± 0.05	0.14 ± 0.02	0.11 ± 0.02
PDK4-KO	3.47 ± 0.68	1.01 * ± 0.10	4.71 * ± 0.48	0.55 * ± 0.06	0.25 * ± 0.03	0.20 * ± 0.02

Table 2 - Post-exercise feeding and nutritional composition in wild-type (WT) and PDK4-knockout (PDK4-KO) mice (n = 8). Values are expressed as mean ± S.E. * Different from WT.

Glycogen concentrations

As a result of exhaustive exercise, muscle glycogen was decreased to 42% of resting concentrations in WT mice and 51% in PDK4-KO mice, with no significant differences between genotypes. After a two-hour recovery period, muscle glycogen restored to resting levels in WT mice and PF mice, and supercompensated in PDK4-KO mice an additional 19% over resting levels ($p<0.05$) (Figure 6).

Following exercise, liver glycogen concentrations decreased significantly to 16% of initial resting values in WT mice and 24% in PDK4-KO mice, again with no differences detected between genotypes. Following recovery, liver glycogen replenished to resting levels in WT and PDK4-KO mice, yet remained at only 49% of resting levels in PF mice ($p<0.001$) (Figure 6).

Metabolite concentrations

Blood glucose was lower in WT mice at exhaustion only, with no differences among groups following recovery (Figure 7). After recovery, blood lactate was lower in PF mice than Rec WT and PDK4-KO mice with no differences between genotypes at other time points. No differences were detected in muscle glucose or G-6-P concentrations between groups, however lactate and alanine were significantly lower in the PF group when compared to Rec WT and PDK4-KO mice (Table 3). In the liver, glucose concentrations decreased as a result of exercise in both genotypes, where after recovery, glucose returned to resting values in PDK4-KO mice, recovered partially in WT mice, and remained at low in PF mice ($p<0.001$) (Table 4).

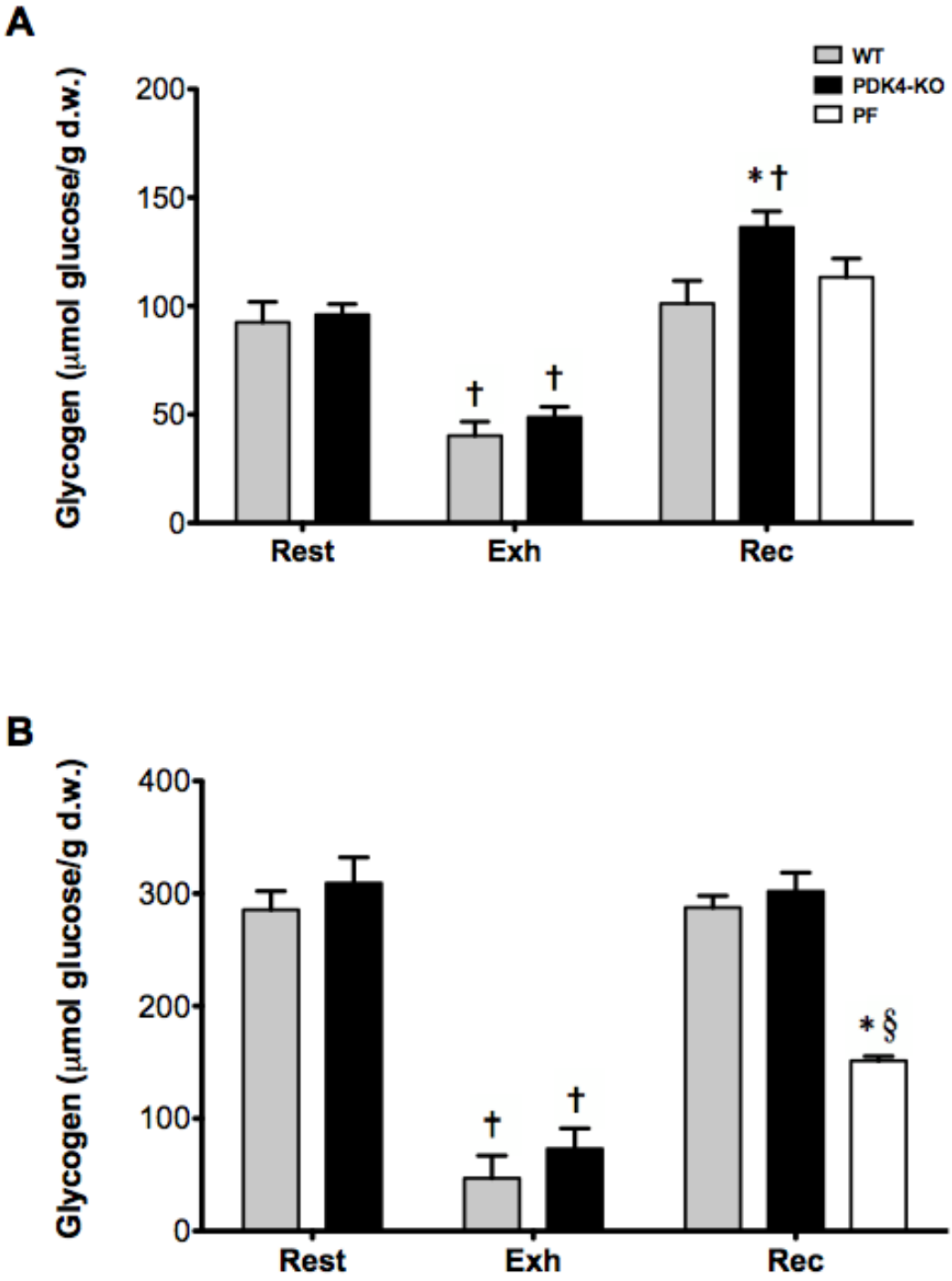

Figure 6 – Glycogen concentrations in (A) muscle and (B) liver at rest, exhaustion (Exh), and recovery (Rec) from exercise (n = 5-8). WT, wild-type; PDK4-KO, PDK4-knockout; PF, pair fed PDK4-KO. Values are presented as mean ± S.E. † Different than rest within a genotype. * Different than WT for a given time-point. § Different than Rec PDK4-KO.

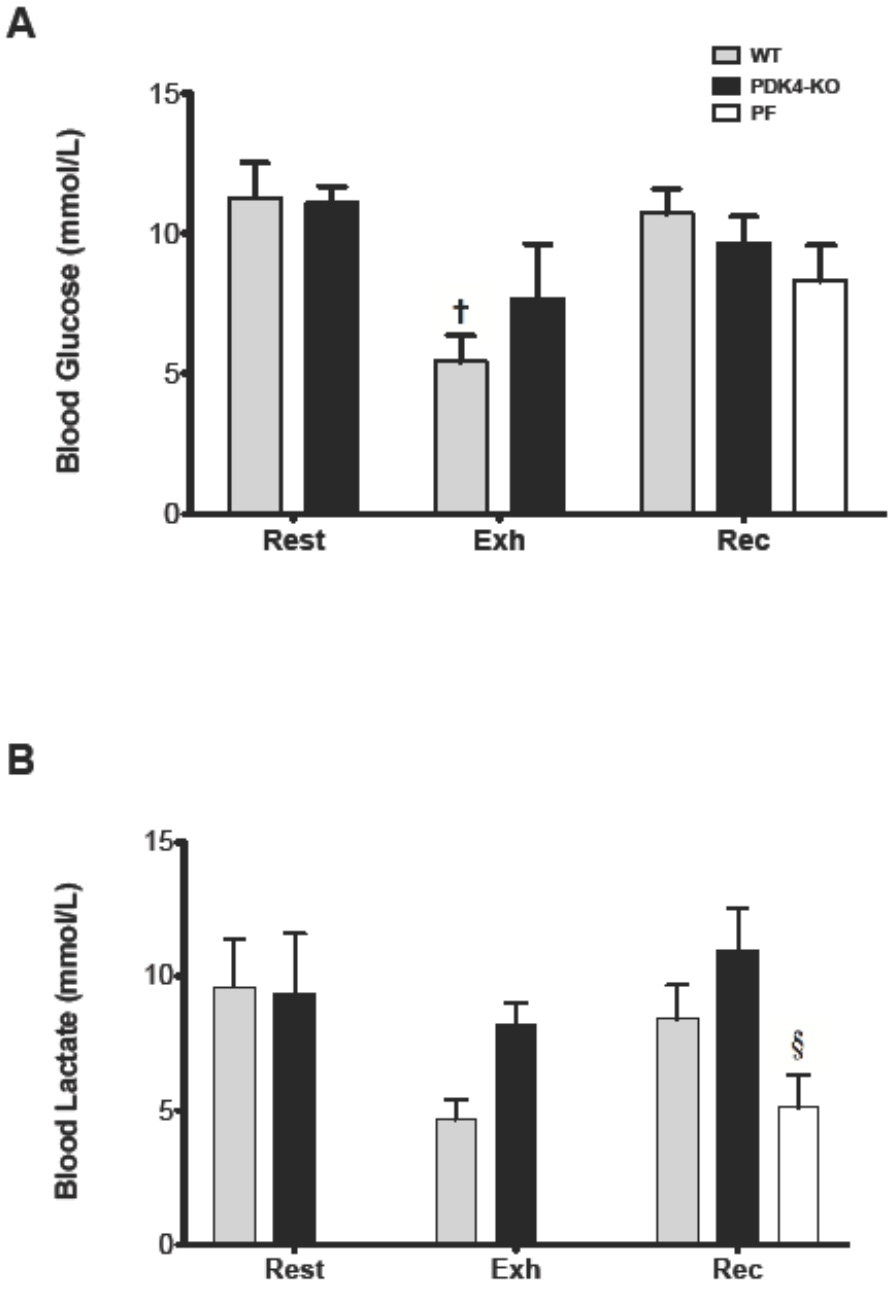

Figure 7 – Blood A) glucose and B) lactate concentrations at rest, exhaustion (Exh) and recovery (Rec) (n = 6-8). WT, wild-type; PDK4-KO, PDK4-knockout; PF, pair fed PDK4-KO. Values are expressed as mean ± S.E. † Different than rest within a genotype. * Different than WT for a given time-point. § Different than Rec PDK4-KO.

	Rest		Exh		Rec		
	WT	**KO**	**WT**	**KO**	**WT**	**KO**	**PF**
Muscle							
Glucose	4.3 ± 1.5	4.7 ± 0.6	2.5 ± 0.8	3.2 ± 0.8	4.6 ± 0.5	3.4 ± 0.5	3.8 ± 0.2
G-6-P	8.1 ± 3.0	8.2 ± 2.7	1.5 = ± 0.7	4.5 ± 1.1	8.5 ± 2.7	7.9 ± 1.7	2.9 ± 0.4
Lactate	23.0 ± 8.6	9.8 ± 4.5	8.0 ± 2.2	8.5 ± 1.1	16.8 ± 5.3	14.7 ± 3.7	3.7 *§ ± 1.5
Alanine	5.4 ± 2.0	4.5 ± 1.6	6.0 ± 1.5	5.0 ± 2.0	4.8 ± 1.1	7.0 ± 1.3	3.0 *§ ± 0.8

Table 3 – Muscle metabolite concentrations at rest, exhaustion (Exh), and recovery (Rec) (n = 5-8). WT, wild-type; PDK4-KO, PDK4-knockout; PF, pair fed PDK4-KO. Values are expressed as μmol/g dry weight ± S.E. † Different than rest within a genotype. * Different than WT for a given time-point. § Different than Rec PDK4-KO.

	Rest		Exh		Rec		
	WT	**KO**	**WT**	**KO**	**WT**	**KO**	**PF**
Liver							
Glucose	86.5 ± 8.8	65.3 ± 9.6	17.3 = ± 0.1	34.4 = ± 9.2	56.0 = ± 7.8	66.6 ± 7.8	27.1 *§ ± 5.1
G-6-P	1.8 ± 0.4	2.2 ± 0.4	0.7 ± 0.1	1.01 ± 0.2	1.4 ± 0.4	2.7 ± 1.1	1.4 ± 0.3
Lactate	31.0 ± 5.0	29.9 ± 7.8	6.7 = ± 3.4	8.2 = ± 2.8	22.1 ± 3.7	29.2 ± 3.1	19.8 ± 7.9
Alanine	8.8 ± 3.3	6.5 ± 2.4	3.6 ± 0.7	11.0 ± 1.5	14.9 ± 3.3	15.8 ± 8.6	12.4 ± 3.7

Table 4 – Liver metabolite concentrations at rest, exhaustion (Exh), and recovery (Rec) (n = 5-8). WT, wild-type; PDK4-KO, PDK4-knockout; PF, pair fed PDK4-KO. Values are expressed as μmol/g dry weight ± S.E. † Different than rest within a genotype. * Different than WT for a given time-point. § Different than Rec PDK4-KO.

Activity of PDHa

Muscle PDHa activity was greater in PDK4-KO mice at Rec than Exh ($p<0.05$), where no differences were detected in WT mice between time points (Figure 8). Liver PDHa activity increased from Exh to Rec in both WT and PDK4-KO mice. PF mice had lower PDHa activity than WT and PDK4-KO mice in both muscle ($p<0.05$) and liver ($p<0.05$).

Protein content of PDH regulatory enzymes

The absence of PDK4 in this study was confirmed in PDK4-KO mice through genotyping liver and muscle tissue through polymerase chain reaction (PCR; Pengfei Wu and Robert Harris, data not shown) as previously described (49). PDK2 and PDP1 protein concentrations were not significantly different between genotypes (Figure 9).

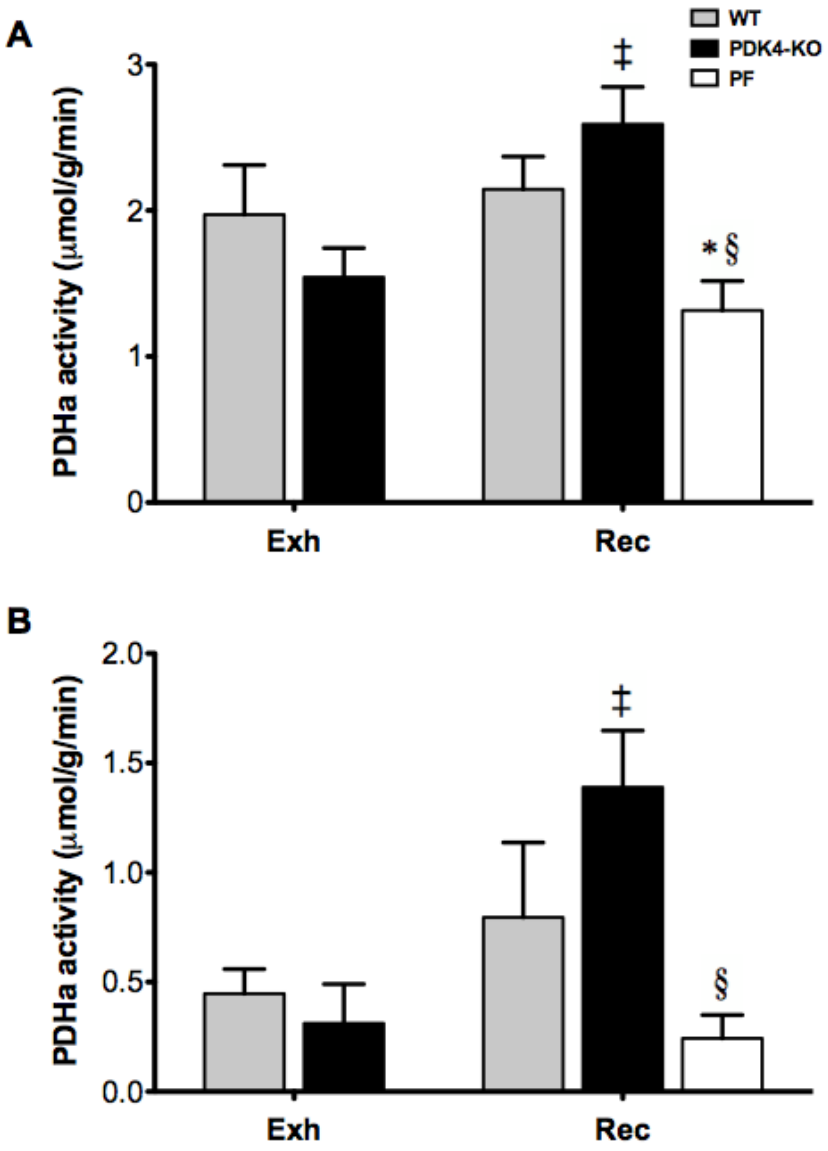

Figure 8 – PDHa activity in A) muscle and B) liver at exhaustion (Exh) and recovery (Rec) (n = 6-8). WT, wild-type; PDK4-KO, PDK4-knockout; PF, pair fed PDK4-KO. Values are expressed as μmol/min/g wet weight ± S.E. ‡ Different than Exh within a genotype. * Different than WT for a given timepoint. § Different than Rec PDK4-KO.

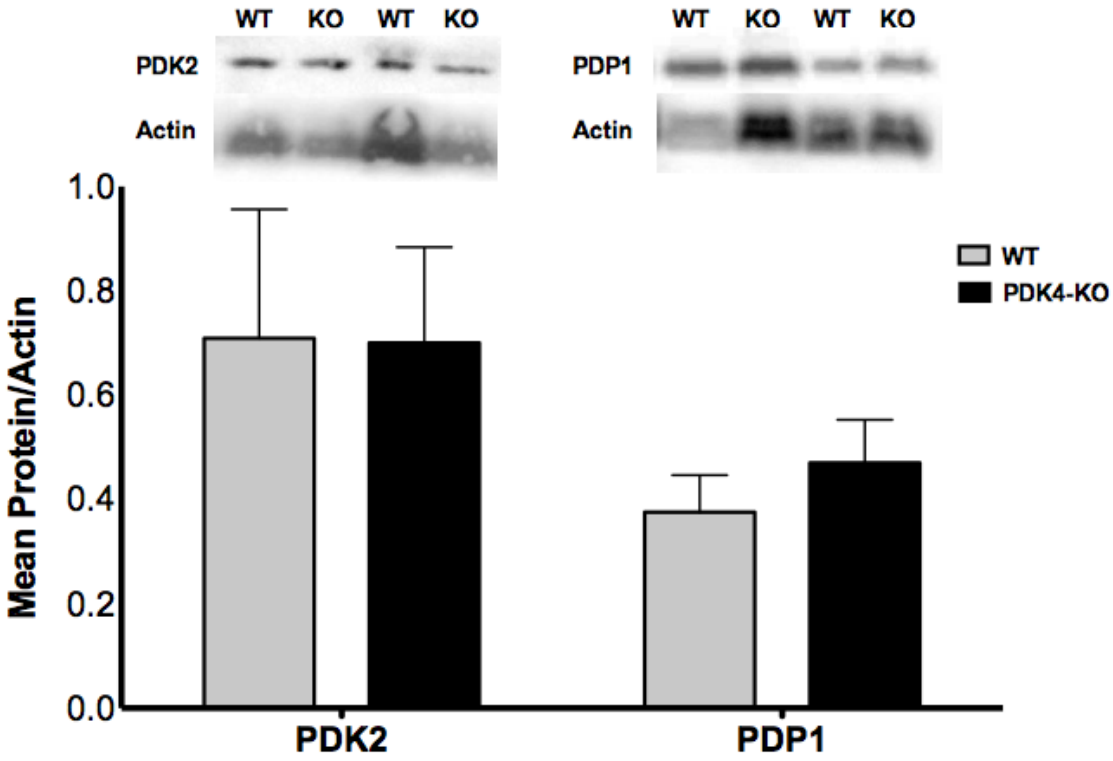

Figure 9 – Muscle protein content of PDK2 (n = 12) and PDP1 (n = 20) of wild-type (WT) PDK4-knockout (PDK4-KO) mice (n=16). Typical blots are shown in inset. Values are expressed as mean protein concentration normalized to actin ± S.E.

Discussion

The present study investigated the involvement of PDK4 in reducing PDHa activity post-exercise and found that 1) PDK4 plays a role during recovery from exhaustive exercise, however, 2) contrary to our hypothesis, the absence of PDK4 does not compromise glycogen resynthesis in skeletal muscle, but 3) causes increased feeding in PDK4-KO mice during recovery, and 4) leads to impaired hepatic glycogen resynthesis in PDK4-KO mice when pair-fed (PF) with WT mice.

In this study, the exercise protocol used was successful in depleting glycogen concentrations in both skeletal muscle and liver. In skeletal muscle, full recovery of glycogen was observed during the two-hour recovery period in WT and PDK4-KO mice and interestingly, the 2-fold greater caloric intake observed in PDK4-KO mice during recovery had provided initial evidence that PDK4 may be involved during exercise recovery. The resulting 1.2-fold supercompensation of muscle glycogen in PDK4-KO mice, however, was unexpected, as we had alternatively hypothesized during experimentation that the greater caloric intake observed during recovery was indicative of greater carbohydrate oxidation through uninhibited PDHa activity, and that PDK4-KO mice would therefore show impaired muscle glycogen resynthesis. Furthermore, when PDK4-KO mice were pair fed (PF) with WT mice, skeletal muscle glycogen fully recovered despite the restriction of calories, which was contrary to our original hypothesis. As PDHa activity was not different between WT and PDK4-KO mice at exhaustion or recovery, and was found to be lower in the PF group, these results would suggest that the absence of PDK4 does not have a direct effect on the resynthesis of skeletal muscle glycogen following exercise.

Although liver glycogen resynthesized to the same degree in recovered WT and PDK4-KO mice, mice in the PF group demonstrated decreased liver glycogen resynthesis. As muscle glycogen recovered fully in these mice, this would suggest that muscle glycogen resynthesis takes priority over glycogen resynthesis in the liver. This is in support of existing evidence demonstrating preference for glycogen resynthesis in skeletal muscle following exercise. For example, rats fasted for 24-hours following exhaustive exercise demonstrated complete recovery of glycogen stores in skeletal muscle, with no recovery observed in the liver during the 24-hour fast (27). Similarly, Maehlum *et al.* (68) also demonstrated increased preference of muscle over liver for the incorporation of glucose into glycogen following glycogen-depleting exercise in humans. Therefore, compromised liver glycogen resynthesis in PF mice in this study emphasizes the priority placed on muscle glycogen synthesis and offers indirect evidence that glucose regulation is impaired in skeletal muscle of PDK4-KO mice, particularly with limited caloric availability. Where WT mice appear to demonstrate a balance between carbohydrate oxidation and lactate and alanine formation in skeletal muscle, PF mice display a reduced ability to form these gluconeogenic precursors, which may indicate a reduced ability to regulate carbohydrate oxidation. Although no significant differences were observed in PDHa activity in skeletal muscle between WT and PDK4-KO mice, more subtle physiological impairments may exist in PDK4-KO mice, which is supported by the reduced formation of lactate and alanine in skeletal muscle of the PF group. This theory would also explain the two-fold increase in caloric intake in PDK4-KO mice fed *ad libitum* during recovery, as greater carbohydrate availability may have been required to produce the same concentration of gluconeogenic precursors (Figure 10).

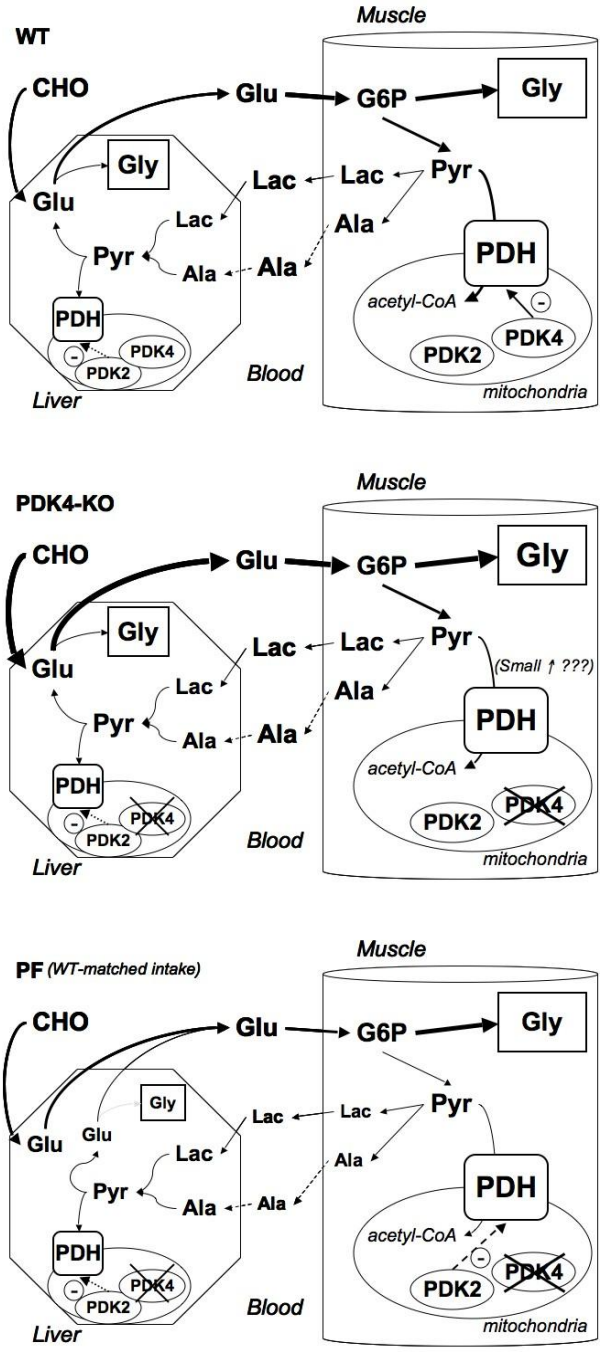

Figure 10 – Summary of findings and suspected pathways in WT, PDK4-KO, and PF mice during recovery from exercise. WT mice (top) represent the expected fates of carbohydrates during recovery, where increased feeding in PDK4-KO mice (middle) when fed *ad libitum* is hypothesized to compensate for potential loss through PDH. PF mice (bottom) have reduced carbohydrate availability for compensation and therefore appear to have reduced gluconeogenic recycling as evidenced by decreased muscle concentrations of glucose and alanine. Abbreviations include glucose (Glu), glucose-6-phosphate (G-6-P), pyruvate (Pyr), lactate (Lac), alanine (Ala), & glycogen (Gly).

It has been previously shown that the PDP isoforms are able to partially override PDK-induced inhibition of PDHa activity during exercise. Kiilerich *et al.* (51) examined the degree of PDHa activation in exercised men fed a high-fat diet and observed that although PDK4 protein expression increased as a result of high-fat feeding, PDHa activity still increased with exercise, but not to the same extent as controls, indicating a reduced ability of PDP to activate the PDH complex. Furthermore, Herbst *et al.* (unpublished data) observed that with fasting, previously shown to increase PDK4 protein expression (113), PDHa activity was not different in incubated EDL muscle of fed and fasted WT mice during stimulation, but was 2-fold greater in PDK4-KO mice. Where the PDPs were able to only partially override the fasting-induced stimulation of PDK4, the absence of PDK4 led to uninhibited PDHa activity. Taken together, the results of these studies suggest that the role of PDK4 during exercise and muscle contraction may be to work antagonistically to the PDPs to regulate small changes in PDHa activity, preventing full activation of PDH. Where PDP stimulation may work to rapidly dephosphorylate PDH, increasing the rate of carbohydrate oxidation, PDK4 may work to phosphorylate and fine-tune the extent to which PDH dephosphorylation may occur, balancing PDHa activity. Relative to the abundantly expressed PDK2 isoform, PDK4 is relatively insensitive to inhibition by pyruvate and has an 8-fold greater specific activity (9), making PDK4 ideal for maintaining a degree of PDH phosphorylation during PDP-stimulated dephosphorylation in the presence of insulin. This effect of fine-tuning PDHa activity was also observed in the present study, as PDHa was greater within PDK4-KO mice at recovery than exhaustion, where this was not true in WT mice, suggesting that the activation of PDH is greater in PDK4-KO mice with feeding. Insulin is a known

stimulator of PDP2 (44) and it is likely that increases in insulin with feeding triggered PDP2 stimulation and therefore increased PDHa activity during recovery. Although it is possible that the differences in PDK4-KO mice may have been due to an insulin response from a 2-fold greater consumption of calories in PDK4-KO mice during recovery, it is also possible that the greater PDHa activation in PDK4-KO mice at recovery is a result of the absence of PDK4 in reducing PDH activation in the presence of PDP stimulation. Hypothetically, increased PDK4 activity during this time would decrease PDHa activity enough to balance carbohydrate oxidation with the production of lactate and alanine, allowing for gluconeogenic recycling and further glycogen resynthesis in both muscle and liver. This theory is supported by previous research that demonstrated reduced glycogen resynthesis and lactate formation during post-exercise recovery with the inhibition of PDK activity by DCA infusion (26). With sufficient availability of food in the present study, PDK4-KO mice would have had similar metabolic properties to PDK4-KO mice in the PF group early in recovery. With limited caloric availability however, food was consumed faster in the PF group than in WT mice, and PF mice would have completed any metabolic processes resulting from feeding by the end of the two-hour recovery period. As muscle glycogen was fully recovered by this time, this would suggest that PDK4 may have greater importance early in recovery, when the initial response to feeding would occur and gluconeogenic recycling appears to be most vital. This would also indicate that PDK4 is not necessary for reducing PDHa activity following repletion of muscle glycogen, as PDHa activity had successfully decreased to resting levels in PF mice.

PDK4 may also play a role in regulating PDHa activity in the liver. Similar to results in skeletal muscle, hepatic PDHa activity increased similarily in WT and PDK4-KO mice from exhaustion to recovery with greater PDHa activity in only PDK4-KO mice between these two time-points. Although PDK4 is believed to be the PDK isoform responsible for decreasing PDHa activity during nutritional perturbations in skeletal muscle, research has previously indicated that this role may fall more predominantly on PDK2 in the liver (102). Glycogen resynthesis in the liver, however, appears to indirectly rely on proper regulation of PDH in skeletal muscle to produce alanine and lactate for gluconeogenic recycling. In the PF group, WT-matched caloric intake and the absence of PDK4 led to decreased production of lactate and alanine in skeletal muscle as well as decreased lactate concentrations in the blood. Reduced availability of these precursors did not affect lactate and alanine concentrations in the liver, however hepatic glucose and glycogen concentrations were lower in the PF group. This suggests that the liver requires greater concentrations of lactate and alanine for sufficient gluconeogenic activity and that liver prioritizes the maintenance of blood glucose concentrations over maintaining hepatic glucose concentrations for glycogen resynthesis.

In summary, this study examined the role of PDK4 in regulating the PDH complex during recovery from glycogen-depleting exercise and provides evidence that PDK4 may be important in fine-tuning the activation of PDH in skeletal muscle to allow for balance between carbohydrate oxidation and gluconeogenic precursor formation, promoting further glycogen resynthesis in both skeletal muscle and the liver.

CHAPTER 5 - General Discussion

This study demonstrated that PDK4 plays a role in regulating carbohydrate metabolism post-exercise, as PDK4-KO mice must intake 2-fold more calories than WT control mice during a two-hour recovery period to adequately recover their glycogen stores. Furthermore, if PDK4-KO mice are pair-fed with WT mice, liver glycogen remains near depletion following the recovery period and both skeletal muscle and blood have lower concentrations of key gluconeogenic precursors. It should be noted that we had hypothesized to see larger differences in PDHa activity in skeletal muscle between genotypes, especially at two-hours of recovery, however the activation state of PDH may have been influenced by other factors, such as the concentration of insulin or the complete resynthesis of muscle glycogen observed in both genotypes. This would suggest that an earlier recovery time-point, perhaps at one-hour, may have provided a better sampling point for observing differences in PDHa activity between genotypes. Previous research has demonstrated sufficient recovery of glycogen in rat skeletal muscle after one-hour of recovery, with only small increases in resynthesis thereafter (11, 87). Pilot data for this study in WT and PDK4-KO mice, however, demonstrated different eating patterns between genotypes during one-hour of recovery (Table 5), and although not statistically different, the absence or presence of eating during recovery could significantly alter the degree of PDH activation by insulin (14, 57, 83) and therefore the rate of carbohydrate oxidation during that period. Furthermore, two-hours of recovery in this study was beneficial in measuring differences in feeding between WT and PDK4-KO mice, which provided evidence that larger caloric consumption following exercise was needed for the same degree of resynthesis in PDK4-KO mice. This observation led to the

inclusion of our PF group, which displayed impaired glycogen resynthesis in the liver, providing greater support on the important role PDK4 plays in skeletal muscle during recovery from glycogen-depleting exercise to favour glycogen resynthesis in both muscle and liver. It would be interesting to examine the same measurements in these mice after one-hour or less of recovery, however without the availability of food, to observe the role PDK4 plays in promoting gluconeogenic recycling of carbohydrates without insulin-stimulation of the PDH complex. The absence of feeding might pose a problem, however, if prolonged, as PDK4 has been shown to be important during fasting (49, 113), which also produces decreased liver glycogen concentrations (43, 49), increased circulation of FFAs (53, 107), and a reliance on PDK4 to decrease PDHa activity (41, 42, 49, 113).

Mouse	WT Intake (g)	KO Intake (g)
1	0.3	0.12
2	0	0
3	0.04	0.02
4	0	0.31
5	0	0.71
Mean ± SD	**0.07 ± 0.13**	**0.23 ± 0.29**

Table 5 – Pilot post-exercise food consumption (grams) in wild-type (WT) and PDK4-KO (KO) mice during 1-hour of recovery after glycogen depleting exercise.

In addition to PDHa activity, glycogen content, and metabolite concentrations, it may have also been beneficial to take blood samples for determination of glycerol and FFA concentrations. In addition to lactate and alanine, glycerol is also a prominent gluconeogenic precursor. Studies using DCA to increase the degree of PDHa activation during exercise recovery in rodents have noticed decreases in lactate, alanine, and glycerol (26), suggesting that this substrate may have been effected in PDK4-KO mice

during recovery. However, where fasting blood lactate and alanine concentrations have been shown to be impaired in PDK4-KO mice after an 18-week high-fat diet, glycerol concentrations contrarily remained consistent with controls (48). As PDK4 was also shown to be necessary in reducing PDHa activity with high-fat feeding, these results further suggest that no changes in glycerol would have been seen in the present study. Previous studies with these mice have demonstrated that PDK4-KO mice have higher circulating concentrations of FFA then WT controls during fasting and high-fat feeding (48, 49). Furthermore, PDK4-KO mice show no changes in the stimuli for FFA mobilization, however they display decreased FFA uptake and oxidation when compared to WT mice (48). As FFAs have been shown to be elevated following exercise (52, 70), it is possible that greater plasma concentrations would be observed in PDK4-KO mice than WT mice at exercise exhaustion and recovery.

In addition to skeletal muscle, the heart also expresses PDK4 in abundance (9) and glycogen concentrations in the heart have been shown to decrease as a result of exercise (19, 98, 99). Therefore the role of PDK4 in recovery of heart glycogen post-exercise was also examined in this study as described for muscle and liver. Although glycogen concentrations did not significantly decrease in either genotype by exhaustion, all recovery groups experienced a ~3.8-fold supercompensation in glycogen concentrations by two-hours (Figure 11). From this it could be theorized that PDK4 does not play an essential role in the heart during exercise recovery, however the heart is one of few tissues to experience a compensatory upregulation from PDK2 in PDK4-KO mice (49) and therefore investigating the role of PDK4 in the hearts of PDK4-KO mice is somewhat problematic. Furthermore, glycogen in the heart is concentrated towards the

conduction system and the bundle of HIS (47), making it necessary to devote whole-heart tissue to the analysis of glycogen. This reduces the number of measurements that can be done on the heart without significantly increasing sample size, leaving the role of PDK4 in the heart during exercise recovery unknown.

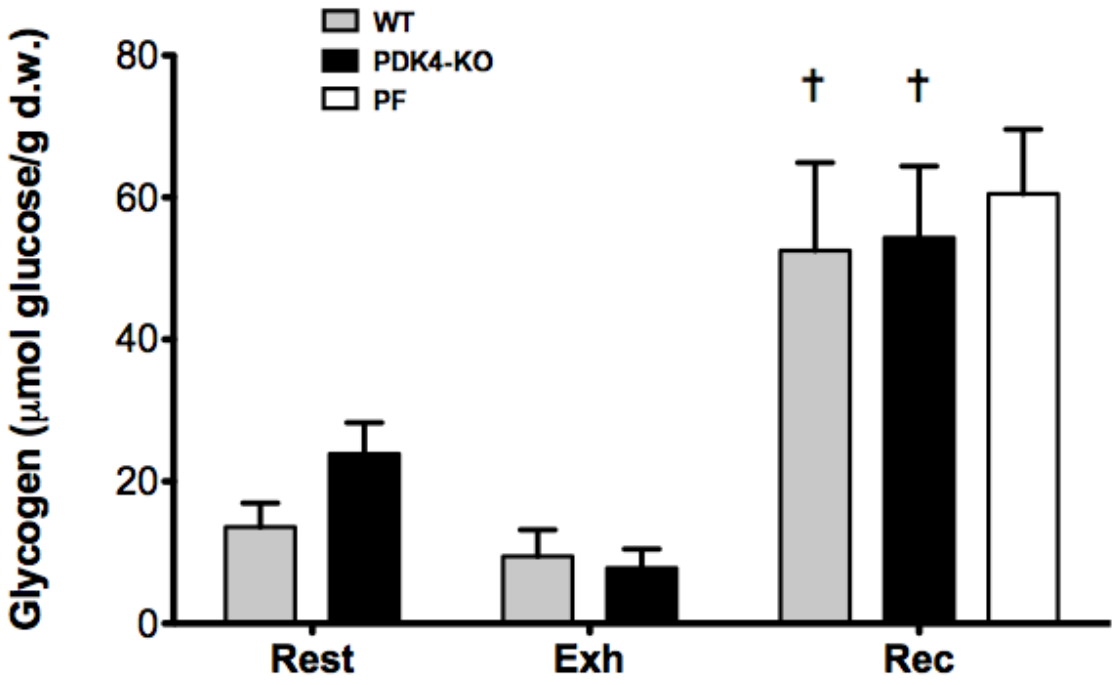

Figure 11 – Glycogen concentrations in the heart at rest, exhaustion (Exh), and recovery (Rec) from exercise (n = 5-8). WT, wild-type; PDK4-KO, PDK4-knockout; PF, pair fed PDK4-KO. Values are presented as mean ± S.E. = Different than rest within a genotype.

Future research should be directed at examining the balance between PDK4 and the PDPs during different exercise and dietary perturbations, focused on isolating the factors that would affect a reduced ability to activate the PDH complex. This effect appears be more prominent with chronic diabetes, as studies have demonstrated increased PDK4 expression (95, 113, 114) along with a decreased capacity to increase PDHa activity (35) in the diabetic state. Furthermore, as this is one of few studies to demonstrate a fine-tuning effect for PDK4 during exercise and recovery, this phenomenon should be further investigated through manipulating the expression of PDK4 and the PDP isoenzymes.

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Appendices

Appendix A – Mouse Chow Composition

Mouse Diet 5015*

DESCRIPTION

Mouse Diet is a complete life-cycle diet specifically designed to support reproduction, growth and maintenance of mice. This diet is formulated using the unique and innovative concept of Constant Nutrition®, paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies. It contains 11% fat to fulfill the metabolic needs of certain mouse strains. Mouse Diet is beneficial in maintaining maximum reproduction for postpartum matings where females are under simultaneous stress of lactation and gestation.

Features and Benefits

- Constant Nutrition® formula helps minimize nutritional variables
- A high-energy diet formulated specifically for all mouse colonies
- Helps maintain maximum reproduction for postpartum matings
- Recommended for mice with low feed intake to improve performance

Product Forms Available

- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")
- Meal (ground pellets), special order

GUARANTEED ANALYSIS

Crude protein not less than	17.0%
Crude fat not less than	11.0%
Crude fiber not more than	3.0%
Ash not more than	6.5%

INGREDIENTS

Ground wheat, dehulled soybean meal, ground corn, wheat germ, brewers dried yeast, porcine animal fat preserved with BHA, calcium carbonate, soybean oil, salt, DL-methionine, dicalcium phosphate, monocalcium phosphate, menadione dimethylpyrimidinol bisulfite (vitamin K), choline chloride, pyridoxine hydrochloride, cholecalciferol, dried whey, vitamin A acetate, lecithin, biotin, dl-alpha tocopheryl acetate, casein, folic acid, vitamin B_{12} supplement, thiamin mononitrate, ferrous sulfate, calcium pantothenate, nicotinic acid, riboflavin, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

FEEDING DIRECTIONS

Mouse Diet should be fed to breeders and lactating females on a free-choice basis. Plenty of fresh, clean water should be available to the animals at all times.

Mice-Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

CHEMICAL COMPOSITION

Nutrients[2]	
Protein, %	18.9
Arginine, %	1.09
Cystine, %	0.28
Glycine, %	0.79
Histidine, %	0.44
Isoleucine, %	0.97
Leucine, %	1.40
Lysine, %	1.05
Methionine, %	0.70
Phenylalanine, %	0.82
Tyrosine, %	0.51
Threonine, %	0.70
Tryptophan, %	0.26
Valine, %	0.92
Serine, %	0.99
Aspartic Acid, %	2.01
Glutamic Acid, %	4.13
Alanine, %	0.99
Proline, %	1.29
Taurine, %	<0.01
Fat (ether extract), %	11.0
Fat (acid hydrolysis), %	11.2
Cholesterol, ppm	28
Linoleic Acid, %	2.05
Linolenic Acid, %	0.17
Arachidonic Acid, %	0.02
Omega-3 Fatty Acids, %	0.21
Total Saturated Fatty Acids, %	3.71
Total Monounsaturated Fatty Acids, %	3.87
Fiber (Crude), %	2.2
Neutral Detergent Fiber[3], %	10.3
Acid Detergent Fiber[4], %	3.0
Nitrogen-Free Extract (by difference), %	52.2
Starch, %	32.5
Glucose, %	0.11
Fructose, %	0.11
Sucrose, %	0.88
Lactose, %	2.70
Total Digestible Nutrients, %	88.1
Gross Energy, kcal/gm	4.68
Physiological Fuel Value[5], kcal/gm	3.81
Metabolizable Energy, kcal/gm	3.70

Minerals	
Ash, %	5.8
Calcium, %	0.80
Phosphorus, %	0.50
Phosphorus (non-phytate), %	0.23
Potassium, %	0.81
Magnesium, %	0.16
Sulfur, %	0.28
Sodium, %	0.44
Chlorine, %	0.71
Fluorine, ppm	7.1
Iron, ppm	170
Zinc, ppm	120
Manganese, ppm	120
Copper, ppm	20
Cobalt, ppm	0.63
Iodine, ppm	1.5
Chromium, ppm	0.46
Selenium, ppm	0.30

Vitamins	
Carotene, ppm	0.20
Vitamin K (as menadione), ppm	3.0
Thiamin Hydrochloride, ppm	13
Riboflavin, ppm	5.6
Niacin, ppm	88
Pantothenic Acid, ppm	20
Choline Chloride, ppm	2000
Folic Acid, ppm	2.9
Pyridoxine, ppm	9.6
Biotin, ppm	0.30
B_{12}, mcg/kg	51
Vitamin A, IU/gm	18
Vitamin D_3 (added), IU/gm	3.3
Vitamin E, IU/kg	66
Ascorbic Acid, mg/gm	—

Calories provided by:	
Protein, %	19.805
Fat (ether extract), %	25.337
Carbohydrates, %	54.858

*Product Code

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

Appendix B – Methods

a. Metabolite Extraction

I. Reagents

0.5 M PCA	21.5 ml 70% PCA Bring to 500 ml with distilled H_20 Store 0-4ºC for 1 month
2.3 M $KHCO_3$	2.3 g $KHCO_3$ Add 10.0 ml distilled H_20 Fresh Daily

II. Procedure

1. Freeze dry tissue (overnight to ensure all water is removed)
2. Store with dry rite in freezer until powdering
3. Tease out connective tissue and powder
4. Place in pre-weighed microcentrifuge tube and weigh (3-5mg)
5. Place tubes in an ice bucket (make sure tubes remain cold)
6. Add 600 µL of pre-cooled 0.5 M PCA
7. Extract for 10 minutes, vortexing several times (ensure all tissue is in contact with PCA)
8. Centrifuge for 10 minutes at 15 000 G (spinning helps remove some of the enzymes that can influence concentration)
9. Remove 540 µL and place in freezer (-20ºC) for 10 min
10. To the frozen supernatant add 135 µL of 2.3 M $KHCO_3$ and vortex until liquid (addition of $KHCO_3$ to a frozen supernatant prevents foaming over)
11. Centrifuge 10 min 0ºC at 15 000G. Remove supernate to assay metabolites.

Dilution Factor = DFa x DFb
DFa = V(PCA)/Muscle Mass (mg) DFb=V(supernatant) + V(KHCO3)/V(supernatant)

b. Hydrolysis of Tissue for Glycogen Assay

A. Reagents

2.0 N HCl	To 83 ml water add 17 ml HCl (37% or 12 N)
2.0N NaOH	To 8 g NaOH add 100 ml water

B. Procedure

1. Use frozen tissue or tissue residue from perchloric acid extract or freeze dried powdered tissue.
2. Add tissue to a microfuge tube (Not to exceed 5 mg dry weight).
3. Add 500 µL 2 N HCl
4. Record weight of each tube
5. Place at 100°C for 2 hours. Mix after the first hour.
6. Re-weigh each tube and add water to achieve initial weight
7. Add 500 µL 2N NaOH, mix well.
8. Store at -90°C until analysis.

Dilution Factor = 1000/ muscle weight (mg)

c. Muscle Glucose & G-6-P/ Glycogen Assay for Plate Reader Fluorometer

HEXOKINASE

Glucose + ATP ----------→ Glucose-6-P + ADP

G-6-P-DH

Glucose-6-P + NADP ----------→ Glucanolactone + NADPH

Reagent	**STOCK CONC**	**FINAL CONC**	**VOLUME 25ml**	**VOLUME 50 ml**	**VOLUME 100ml**
1. Tris (on shelf) **(pH 8.1)** stored in fridge	1.00 M	50 mM	1.25 ml	2.5 ml	5.00 ml
2. $MgCl_2$ (on shelf) make fresh (.2033g/ml)	1.00 M	1 mM	25 µL	50 µL	100 µL
3. DTT (found in -20) stored in aliquots (-80)	0.5 M	0.5 mM	25 µL	50 µL	100 µL
4. ATP (found in -20) stored in aliquots (-80)	300 mM	300 µM	4.14 mg	8.27 mg	16.54 mg
5. NADP (found in -20) stored in aliquots (-80)	50 mM	50 µM	25 µL	50 µL	100 µL
6. G-6-P-DH found in fridge (Sigma G-5760)	2660 U/ml	0.02 U/ml	See	Procedure	Below
7. Hexokinase found in fridge (Sigma L-5500)	1338 U/ml	0.14 U/ml	See	Procedure	Below

Note: Mix reagents 1-5 together. Bring to volume with distilled water and adjust to pH 8.1.

Preparation of Dilute Enzyme

1. Add 1.5 uL of G-6-P-DH to a test-tube containing 1.5 ml of Reagent. Mix by inversion.

2. Add 5.2 uL of Hexokinase to a test tube containing 1 ml of reagent. Mix by inversion.

Procedure for Assay

Part 1.

1. Fill three wells with a blank (10.00 µL dH_2O per well)
2. a. Vortex each concentration mixture before pipetting
   b. Fill the next five wells with 10.00µL of varying concentrations of 5µM/ 2.5 µM Glucose standard (0.1mM, 0.2 mM, 0.4 mM, 0.8 mM, 1.2 mM)
3. a. Vortex each concentration mixture before pipetting
   b. Fill the next five wells with 10.00µL of varying concentrations of 5uM G6P standard (0.1mM, 0.2 mM, 0.4 mM, 0.8 mM, 1.2 mM) (only if determining G6P-DH)
4. a. Vortex each sample before pipetting
   b. Add 10.00 µL of sample to the appropriately wells
5. Add 185 µL of buffer to each well
6. Incubate for 15 minutes
7. Read the plate at a sensitivity of 95 (excitation setting 340, emission setting 460) (base line reading)

Part 2.

1. Add 5 µL of dilute G-6-P-DH to all of the wells
2. Place in the dark for 17 minutes
3. Read the plate (excitation setting 340, emission setting 460)

Part 3.

1. Add 5 µL of dilute Hexokinase to all of the wells
2. Place in the dark for 20 minutes
3. Read the plate (excitation setting 340, emission setting 460)

Note: Everything analyzed in triplicate

Glucose Standard Curve (for glycogen)
2.5 mM stock (25 mg of glucose (Sigma G 8270) to 55.2 ml of dH_2O)

Conc (mM)	Stock (uL)	dH_2O (umol•L^{-1})
0.1	40	960
0.2	80	920
0.4	160	840
0.8	320	680
1.2	480	520

Glucose Standard Curve (for glucose) / G-6-P Standard Curve (for metabolite-extraction analysis only)
2.5 mM glucose stock (25 mg of glucose (Sigma G 8270) to 55.2 ml of dH_2O)
2.5 mM G-6-P stock (42.04mg of G-6-P (Sigma G7375) into 50ml of dH_2O)

Conc (mM)	Stock (μL)	dH_2O ($\mu mol \bullet L^{-1}$)
0.0125	5	995
0.025	10	990
0.05	20	980
0.1	40	960
0.2	80	920
0.3	120	880

d. Muscle Lactate Assay for Plate Reader Fluorometer

$$\text{Lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + NADH + H^+$$

$$\text{PYRUVATE} + \text{Hydrazine} \rightarrow \text{Pyruvate Hydrazone}$$

Reagent	STOCK CONC	FINAL CONC	VOLUME 25ml	VOLUME 50 ml	VOLUME 100ml
1. Hydrazine (on shelf) *stored in fridge*	1.00M	100.0 mM	2.5 ml	5.00 ml	10.00 ml
2. Glycine (on shelf) *stored in fridge*	1.00 M	100.0 mM	2.5 ml	5.00 ml	10.00 µL
3. NAD^+ (found in -20) *stored in aliquots (-80)*	50.0 mM	0.5 mM	250 µL	500 µL	1000 µL
4. LDH (found in fridge) Sigma L-2500	13110 U/ml	8 U/ml	See	Procedure	

Note: Mix reagents 1-3 together. Bring to volume with distilled water and adjust to pH 10.

Preparation of Dilute Enzyme

Add 17.25 µL of LDH to 1.0 ml of reagent. Mix by inversion.
(use 60 µL if using L-5132, LDH)

Procedure for Assay

Part 1.

1. Fill three wells with a blank (10.00 µL dH_2O per well)

2. a. Vortex each concentration mixture before pipetting
   b. Fill the next five wells with 10.00µL of varying concentrations of lactate standard (see below)

3. a. Vortex each sample before pipetting
   b. Add 10.00 µL of sample to the appropriately wells

4. Add 185 µL of buffer to each well
5. Incubate for 15 minutes

6. Read the plate at a sensitivity of 100 (excitation setting 340, emission setting 460) (base line reading)

Part 2.

1. Add 10 μL of dilute LDH to all of the wells

2. Place in the dark for 120 minutes

3. Read the plate (excitation setting 340, emission setting 460, sensitivity 100)

Note: Run everything in triplicate

Lactate Standard Curve
Pre-made lactate standard (4.44 mM)

Conc (mM)	Stock (uL)	dH_2O ($umol \bullet L^{-1}$)
0.1	23	977
0.2	45	955
0.4	90	910
0.8	180	820
1.2	270	730

Conc (mM)	Stock (uL)	dH_2O ($umol \bullet L^{-1}$)
0.025	5.6	994
0.05	11.25	988
0.1	22.5	978
0.2	45	955
0.4	90	910
0.8	180	820

e. Muscle Alanine Assay for Plate Reader Fluorometer

Alanine Aminotransferase

Alanine + 2-Oxogluterate ----------→ Pyruvate + Glutamate

LDH

Pyruvate + NADH + H^+ ----------→ Lactate + NAD^+

Reagent	STOCK CONC	FINAL CONC	VOLUME 25ml	VOLUME 50 ml	VOLUME 100ml
1. Tris HCl	1.00 M	25 mM	0.625 mL	1.25 mL	2.5 mL
2. Tris Base	1.00 M	25 mM	0.625 mL	1.25 mL	2.5 µL
3. 2-Oxogluterate Sigma K3752 ***Make fresh 190mg/ml***	10.0 mM	50 µM	125 µL	250 µL	500 µL
4. NADH Sigma N-8129 ***make fresh 3.6 mg/ml***	5.0 mM	15 µM	75 µL	150 µL	300 µL
5. LDH Sigma L2500	13110 U/ml	0.25 U/ml	0.5 µL	1 µL	2 µL
6. Alanine Sigma G9880					
7. Glutamic-Pyruvic Transaminase (GPT) Sigma 9880	15.1mg protein/ml; 88units/mg protein	5 U/ well			

Note: Mix reagents 1-2 together, bring to volume with distilled water, adjust pH to 8.1, and add reagents 3-5.

Preparation of Dilute Enzyme

Add 188.14 µL of GPT (Sigma G9880) to 1mL of reagent. Mix by inversion.

Procedure for Assay

Part 1.

1. Fill three wells with a blank (10.00 μL dH_2O per well)

2. a. Vortex each concentration mixture before pipetting
   b. Fill the next five wells with 10.00μL of varying concentrations of the alanine standard (see below)

3. a. Vortex each sample before pipetting
   b. Add 10.00 μL of sample to the appropriate wells

4. Add 185 μL of buffer to each well
5. Incubate for 30 minutes
6. Read the plate at a sensitivity of 100 (excitation setting 340, emission setting 460) (base line reading)

Part 2.

1. Add 10 μL of dilute GPT to all of the wells

2. Place in the dark for 2 hours

3. Read the plate (excitation setting 340, emission setting 460, sensitivity 100)

Note: Run everything in triplicate

Alanine Standard Curve

2.5 mM alanine standard: dissolve 44.55mg of alanine into 200ml dH_2O.

Conc (mM)	Stock (μL)	dH_2O ($\mu mol \bullet L^{-1}$)
0.0125	5	995
0.025	10	990
0.05	20	980
0.1	40	960
0.2	80	920
0.3	120	880

f. Determination of PDH Activity

(1) PDH Assay Buffer (for both active and total determinations)

Reagent	M.W.	[final], mM	Amt/200 mL
Tris	121.1	144.4	3.4974 g
EDTA	372.24	0.72	0.0536 g
MgCl2	203.3	1.44	0.0586 g

pH to 7.8 with HCl, and freeze in 4 ml aliquots at -20°C.

(2) PDHa Homogenizing Buffer

Reagent	M.W.	[final], mM	Amt/100 mL
Sucrose	342.3	200	6.8460 g
KCl	74.55	50	0.3728 g
MgCl2	203.3	5	0.5083 g
EGTA	380.4	5	0.1902 g
Tris HCl	121.1	50	0.6055 g
NaF	41.99	50	0.2100 g
DCA	12.1 M stock	5	41.3 ul
Triton X-100	0.10 ml	0.1% (v:v)	0.10 ml

pH to 7.8 with NaOH and freeze in aliquots at -20°C

3) PDHt Homogenizing Buffer (**cannot be used with resting samples)

Reagent	M.W.	[final], mM	Amt/100 mL
Sucrose	342.3	200	6.8460 g
KCl	74.55	50	0.3728 g
MgCl2	203.3	25	0.5083 g
EGTA	380.4	5	0.1902 g
Tris HCl	121.1	50	0.6055 g
Glucose	180.16	10	0.1802 g
DCA	12.1 M stock	5	41.3 ul
CaCl2	147.02	10	0.1470 g
Triton X-100	0.10 ml	0.1% (v:v)	0.10 ml

pH to 7.8, and freeze in aliquots at -20°C.
Add hexokinase daily (sulphate free); prepare 820 U/ml stock and store at -20°C.
Stable for 30 d of repeated freezing and thawing.

**Note: 1) NaF, phosphatase inhibitor; 2) DCA, kinase inhibitor; 3) Triton, digest membrane phospholipids to facilitate release of membrane-bound PDH; 4) $CaCl_2$ and*

$MgCl_2$, provide divalent ions for phosphatase activation; 5) glucose and hexokinase, elimination of endogenous ATP.
Homogenization Procedure (active and total):

N.B. Prior to homogenization, try to dissect each sample free of all connective tissue and dried blood, etc. Total Cr content should be measured from each homogenate, and all values for a subject corrected to the highest Cr value. Note that the picric acid method of determining total Cr is **not suitable** for these homogenates. See below for total Cr determination.

1. Place 100 μl of homogenizing buffer (use the appropriate buffer, for either the transformed (active) or total activity) into a homogenizing mortar, depending on sample size. NOTE: total PDH activity is not measurable on resting samples.

2. Place the tube in small Styrofoam block, place all on a weighing balance and tare it.

3. Add 5-10 mg of wet, frozen muscle to the tube, and record its weight (mg).

4. Multiply weight of muscle by 30, then subtract 100 from this total (for the 100μl of buffer that has already been added) and add the remaining amount of cold homogenizing buffer (kept on ice) to the sample in the mortar.
(eg. 10 mg muscle sample – 30X10 mg = 300μl to be added – 100 μl already added = 200 μl more to be added)

5. Begin homogenization of the sample with gentle twisting hand movements (on ice). NOTE: the time between weighing and homogenizing should be kept to a minimum.

6. Thereafter, homogenize for 50s with a motor-driven glass pestle at ~20-30 rpm (on ice).

7. Store the homogenate in an eppendorf tube (with small hole in top) in liquid N_2 and analyze for PDH activity (within 1-2 d). *It is very important to immediately "snap-freeze" the homogenates in liquid N_2 to prevent reformation of vesicles.*
Approximately 50% of these reformed vesicles will be "inside-out", masking the PDH complex within the vesicle and resulting in PDH activities of ~50% of that expected. Similarly thaw samples one at a time, just prior to analysis to prevent vesicle formation at room temperature.

8. After completion of PDH measurements, the remaining homogenate should be kept at -80°C for total Cr determination.

Determination of total Cr.

1. Thaw homogenates at room temperature; vortex.

2. Add 30 μl of homogenate to 30 μl of 0.5 N PCA with (with EDTA).

3. Vortex well, and let sit at room temperature for 60 min (PCr is hydrolyzed to Cr at this stage).

4. Neutralize with 7.5 μl of 2.2 M $KHCO_3$, vortex, and centrifuge to separate salt from the supernatant.

5. Measure Cr photometrically with enzymatic method. If desired, PCr can be measured on a few randomly chosen samples to insure that its hydrolysis was complete.

Determination of PDH Activity (active and total)

1) Preparation of PDH Reagent (choose (A) or (B) below):
(A) For PDHa activity in frozen muscle samples, for a given sample (homogenate), there are duplicate (2) incubations for the determination of PDH activity, plus 1 blank (total of 3 incubations). Assume a requirement of 0.72-1.0 ml of reagent* mixture per incubation when preparing solutions.

Reagent Mixture*	M.W. (g/mol)	Stock, mM	Stock, mg/mL	Vol. for 12 incubations	Vol. for 18 incubations	Vol. for 24 incubations	Vol. for 30 incubations
PDH Assay Buffer				9 mL	13.5 mL	18 mL	22.5 mL
NAD	717.4	39	28	1 mL	1.5 mL	2 mL	2.5 mL
CoASH	767.6	13	10	1 mL	1.5 mL	2 mL	2.5 mL
TPP	460.8	13	6	1 mL	1.5 mL	2 mL	2.5 mL
TOTAL				12 mL	18 mL	24 mL	30 mL
Pyruvate**	110	26	3.1	0.5 ml	0.75 ml	1.0 ml	1.25 ml

* Reagent mixture is comprised of PDH assay buffer, NAD, CoASH and TPP.
**Pyruvate is added to initiate the reaction through PDH.

(B) For PDHa determination as part of a PDK Assay:
- PDH measurements as part of the PDK assay need one to be done in duplicate (blank is always zero). For these assays, afford at least 0.26 ml of reagent per incubation.

Reagent Mixture*	M.W. (g/mol)	Stock, mM	Stock, mg/mL	Vol. for 16 Samples***	Vol. for 24 Samples***	Vol. for 32 Samples***
PDH Assay Buffer				8 mL	12 mL	16 mL
NAD	717.4	39	28	0.9 mL	1.35 mL	1.8 mL
CoASH	767.6	13	10	0.9 mL	1.35 mL	1.8 mL
TPP	460.8	13	6	0.9 mL	1.35 mL	1.8 mL
TOTAL				10.7 mL	16.05 mL	21.4 mL
Pyruvate**	110	26	3.1	0.5 ml	0.75 ml	1.0 ml

* Reagent mixture is comprised of PDH assay buffer, NAD, CoASH and TPP.
** Pyruvate is added to initiate the reaction through PDH.
***16 samples (for example) indicates 2 biopsies x 8 PDHa's/biopsy x duplicate = 32 incubations

2) Incubation of Samples:

1. Pre-heat block heater to 37°C. Monitoring of the temperature may be facilitated by the placement of a thermometer sealed by parafilm in a tube of water, which is then left in one of the holders of the block heater for the duration of the incubations.

2. For each sample (homogenate), the reaction will be run in duplicate (initiated with pyruvate) and with one blank (initiated with water). To determine the reaction rate, acetyl-CoA production will be determined at the precise intervals of 1, 2 and 3 min after the initiation of the reaction. Therefore, use coloured eppendorf tubes (1 (white), 2 (green), and 3 (blue) min) for each of the duplicates (A, B) and blank (C), if necessary. e.g. the 3 min determination of duplicate B would be a blue tube labeled, "B" along with the sample information. **note: there are no blanks run with PDK assays**

3. Pipette 40 µl of 0.5 N PCA into each of the labeled tubes and place in a separate rack.

4. Pipette correct amount of reagent into 12x75 culture tubes in block heater and allow it to warm for sufficient time (at least 3 incubations). Be sure to warm more as you proceed with the PDHa's! The pyruvate (~150 µl) should also be warmed in the block heater for at least a period of 3 incubations, but in a separate and distinct tube.

5. **A)** For a PDHa Assay, add 30 µl of the homogenate (using a positive displacement pipetter) into 720 µl of pre-warmed reagent mixture. Mix well by drawing up and ejecting from the pipetter several times, or by vortexing *(very gently (foaming indicates denaturing of the protein.).*

B) For a PDK Assay, add 15 μl of the homogenate with a positive displacement pipetter into 260 μl of pre-warmed reagent mixture. Mix well by drawing up and ejecting from the pipetter several times.

6. **A)** For a PDHa Assay, initiate the reaction by adding 30 μl of pyruvate. Vortex *very gently* and start the timer. It is not crucial that the timer is started immediately upon the addition of pyruvate (i.e. within a few seconds). It is extremely important, however, than once the timer has begun, samples of the incubating solution are obtained at *precisely* timed 1 min intervals, for determination of the slope (reaction rate). Variability in the exact starting time of the reaction will merely cause slight deviations in the y-intercept from 0.
   B) For a PDK Assay, follow the same procedure, but start with only 15 μl of pyruvate.

7. **A)** For a PDHa Assay, after precisely 1 min, draw 200 μl of the incubation mixture and pipette into the appropriately labeled eppendorf tube containing PCA. Vortex, and let sit at room temperature until the entire incubation trial is complete.
   B) For a PDK Assay, after precisely 1 min, draw 70 μl of the incubation mixture and pipette into the appropriately labeled eppendorf tube containing PCA. Vortex, and let sit at room temperature until the entire incubation trial is complete.

8. Repeat step #7 at precisely 2 and 3 min into the incubation.

9. Repeat steps #5 to #8 so that the entire procedure is run in duplicate.

10. Run a blank when necessary for each homogenate by repeating steps #5 to #8, substituting 30 μl of water for pyruvate.

11. Neutralize samples with 10 μl of 1 M K_2CO_3 to each of the tubes and vortex. Allow the acidified homogenates to sit for 5 min before neutralization to fully stop the reaction.

12. Store neutralized extracts at -80°C until analyzed for acetyl-CoA content. Acetyl-CoA is viable for ~1 wk at this temperature. Prior to analysis, thaw samples and vortex.

13. NOTE: Incubation of duplicates and blank can be run simultaneously, if the initiation of the reactions are staggered such that the sampling times of each (@ 1, 2, and 3 min) are separated by ~15 - 20 s.

14. Use the following dilutions for acetyl-CoA determinations:

    a) human PDH_{tot} and PDH_a (exercise): 10 μl extract + 190 μl water
    b) human PDH_a (rest): 20 μl extract + 200 μl water

f. Herbst's Guide to Western Blotting

Sample Preparation:

Homogenization

1. Make up homogenization buffer:
    - 250mM sucrose = 21.4g
    - 100mM KCl = 1.865g
    - 5mM EDTA = 0.4695
    - add 225mL dH_2O, fix pH 6.8, top up to 250mL dH_2O and mix well, store in fridge
2. Remove wanted buffer and add protease inhibitor tablet (from antibody drawer) (Roche, 1 tablet /10mL) and add phosphatase inhibitor (Roche, 1 tablet /10mL)
3. Prepare glass homogenizers with ~ 200µL of buffer, tear in scale, remove ~ 10mg chunk of muscle from sample for analysis, add muscle and weigh
    - keep on ice when possible
4. Correct buffer volume in homogenizer so that 10µL of buffer is present for every 1mg of sample
5. Homogenize well on ice

Protein Determination:

1. Make up or remove BSA standard (1mg/ml) from -20
2. Make up standard:

Final Protein Concentration (mM)	**Volume of BSA (µL)**	**Volume of dH_2O (µL)**
1	leave as is	0
0.5	500 from '1'	500
0.25	500 from '0.5'	500
0.125	500 from '0.25'	500
0.05	100 from '0.5'	900
0	0	Leave as is

3. Pipette everything in triplicate in a clear microtitre plate. Pipette the samples as 9μL of dH_2O and 1μL of sample for whole homogenate (10x dilution), 9.5μL of dH_2O and 0.5μL of sample for mitochondrial suspension (20x dilution).
4. Add 200μL of diluted buffer (1:4 with Bio-Rad Protein Assay Dye Reagent : dH_2O)
5. Let incubate for 5min and read absorbance as wavelength 595nm
6. In excel run polynomial curve with blanks subtracted, multiply determined y-intercept by 10 to determine protein concentration for whole homogenates, by 20 for mitochondrial isolations.
7. To prep samples for blotting[1] (best done on same day as SDS PAGE):

Total Volume (TV)	Optional 25 - 100 μL
Sample Volume (SV)	$\mathrm{SV} = \mathrm{T}V \bullet \frac{[\text{Desired Final Protein}]}{[\text{Protein}]}$
Sample (Laemelli) Buffer (SB)	$SB_f = \frac{TV}{[SB_i]}$ (i.e. $\frac{\mathrm{TV}}{5}$ for 5x $\mathrm{SB_i}$)
Water (dH_20)	$dH_2O = TV - SV - SB$
1M DTT [2]**	= 10% of total volume

SDS PAGE Procedure:

Electrophoresis Preparation

1. Before using glass plates, wipe with methanol and a kimwipe
2. Assemble the glass-holding apparatus with the short plate facing front. Ensure plates are flush and level before locking and inserting onto the gel casting stand
3. Prepare gels (makes 2):

Percent Gel	**dH_2O (ml)**	**Protogel (ml)** (30% acrylamide)	**Gel Buffer (ml)**
4 (Stacking)	**6.1**	**1.3**	**2.5 Stacking**
6	5.3	2.0	2.5 Resolving
8	4.62	2.67	2.5 Resolving
10 (Running)	**3.96**	**3.33**	**2.5 Resolving**

[1] If [protein] is <1, cannot reliably perform blotting

[2] DTT is optional. Effects redundant with β-mercaptoethanol (in Laemelli buffer)

12	3.29	4.0	2.5 Resolving
15	2.29	5.0	2.5 Resolving

4. Before pouring the **running gel**, add 100μL APS (60mg/600μL) and 10μL of TEMED. Swirl and pour until reaching the depth of the comb. Use methanol to remove any bubbles and dump once hardened
5. Before pouring the **stacking gel**, add 100μL APS and 20μL TEMED. Swirl and pour until the top of the short plate is reached. Insert combs of desired size. Once hardened, remove combs and rinse with dH_2O
6. Place gel cassettes into electrode assembly with the short plates facing inwards, clamp down firmly and place into mini-tank
7. Boil samples for 5-minutes and place subsequently on ice for 5-minutes while preparing *running buffer* (50mL 10x running buffer + 450mL dH_2O)
8. Fill the inner chamber of the apparatus with diluted running buffer add ~200mL to the outside chamber
9. Load the standard[3] and the appropriate amount of sample into each well. (Note: to determine the appropriate amount of sample to load, perform a separate optimization with varying amounts of protein. 5-10μL may be used successfully if just practicing).
10. Run electrophoresis at 120-volts for 90-minutes or until the blue dye travels to the bottom edge of the plate. If tiny bubbles rise then electrophoresis is working[4]. Prepare transfer buffer.

Transfer

1. Remove gels carefully, discard the stacking gel, and transfer to a plastic try to equilibrate in transfer buffer

[3] Lui says: "5μL works nicely"
[4] Thing's aren't going well if:
- the amount of buffer inside the tank is going down (you have a leak!)
- the bands form a "sad face" (you may be running the gel at too high a voltage or have a leak)

2. Create the "transfer sandwich":
    - white/clear side of the holder
    - sponge/ fibre pad (presoaked in transfer buffer)
    - filter paper (presoaked in transfer buffer)
    - membrane (PVDF presoaked in methanol)
    - Gel
    - filter paper (presoaked in transfer buffer)
    - sponge/ fibre pad (presoaked in transfer buffer)
    - black side of the holder

    *Be sure to roll out any air bubbles, close firmly, and place the black side of the holder to the black (/back) side of the electrophoresis apparatus.
3. Fill the tank with transfer buffer and the surrounding environment with plenty of ice to prevent from cooking the gel and add a stir-bar to help with heat loss.
4. Run the transfer at 100-volts for 60-minutes

Antibodies

1. Discard the gel and filter papers and transfer the membranes to small dishes
2. Block with 20mL TBST-5% skim milk[5] or TBST-3% BSA solution (4.5g skim milk powder in 90mL) for 1-hour
3. Discard blocking solution and add 1^{o} AB in TBST-5% skim milk, incubating for 1-hour or overnight[6] with the appropriate dilution

Antibody	Size (kDa)	Dilution	Amount (μL in 10mL)
PDK 1-4	46-48	1:200	50[7]
PDP1c	53	1:3000	3.3
PDP2	60	1:3000	3.3
PDH E1α	41	1:5000	2
PDH E2	52	1:5000	2
PDH E2/E3bp	50	1:1000	10

[5] Can substitute with a TBST-3% BSA solution (3.0g BSA in 100mL TBST)
[6] McMeekin says: "overnight works better"
[7] Dunford used 5μL in 10mL successfully

CS	50	1:500	20
COX (subunits 2-4)	20-26	1:000	10
ANT	30	1:200	50[8]

4. Wash 3-5x 5-minutes with TBST (10mL)
5. Add 2^{o} Ab (dependent on 1^{o} Ab) in TBST-5% skim milk solution for 1-hour with the appropriate dilution

Secondary	**Dilution**	**Amount** (μL in 10 mL)
Anti-mouse	1:5000	2
Anti-goat	1:20000	0.5
Anti-rabbit	1:10000	1

Enhanced Chemiluminesence

1. Wash 3x 5-minutes with TBST [9]
2. Combine 1mL of each chemiluminescent HPR substrate (peroxide solution + luminal reagent) over membrane and continue to pipette over membrane or let rock for 5-minutes
3. Place membrane protein-side down on glass and cover back with parafilm
4. Take an initial photo with a pin beside the standard that represents the weight of your protein for overlapping later if need be. Take a subsequent photo with the appropriate exposure time and analyze later with "Imagej".

[8] Herbst used 10μL in 10mL successfully
[9] Herbst says: In some instances, longer incubations in TBST produce cleaner blots (PDKs especially)

Reagents:

Homogenizing Buffer
- 250mM sucrose (21.4g)
- 100mM KCL (1.865g)
- 5mM EDTA (0.4695g)
- add 225mL dH_2O, fix pH 6.8, top up to 250mL dH_2O
- mix and store in fridge

Laemelli Buffer (2x Sample Buffer)
- 2.5ml 0.5M Tris-HCl pH 6.8
- 2ml glycerol
- 2ml 10% SDS
- 0.2mL 1% bromophenol blue
- 1ml β-mercaptoethanol
- 0.3mL dH_2O

Running Gel Buffer
- 1.5M Tris-HCl, pH 8.8

Stacking Gel Buffer
- 0.5M Tris-HCl, pH 6.8

10x Running Buffer, pH 8.3
- 250mM Tris base
- 1.92M glycine
- 1% SDS
- Store at 4^oC. If precipitation occurs, warm to room temp before use.

10x Transfer Buffer (semi-dry)
- 312.5mM Tris base (3.786g)
- 2.4M glycine (18g)
- fill to 100mL with dH_2O
- *before use, dilute 80mL in 720 mL dH_2O and add 200mL of methanol

10x TBS
- 200mM Tris base
- 1.37M NaCl
- 38mL of 1M HCl/L of TBS made
- adjust to pH 7.5

TBST
- dilute 100mL of 10x TBS in 900mL dH_2O
- add 4mL of 25% Tween 20 while stirring (polyoxyethylenesorbitan monolaurate)

Appendix C – Western Blot Results

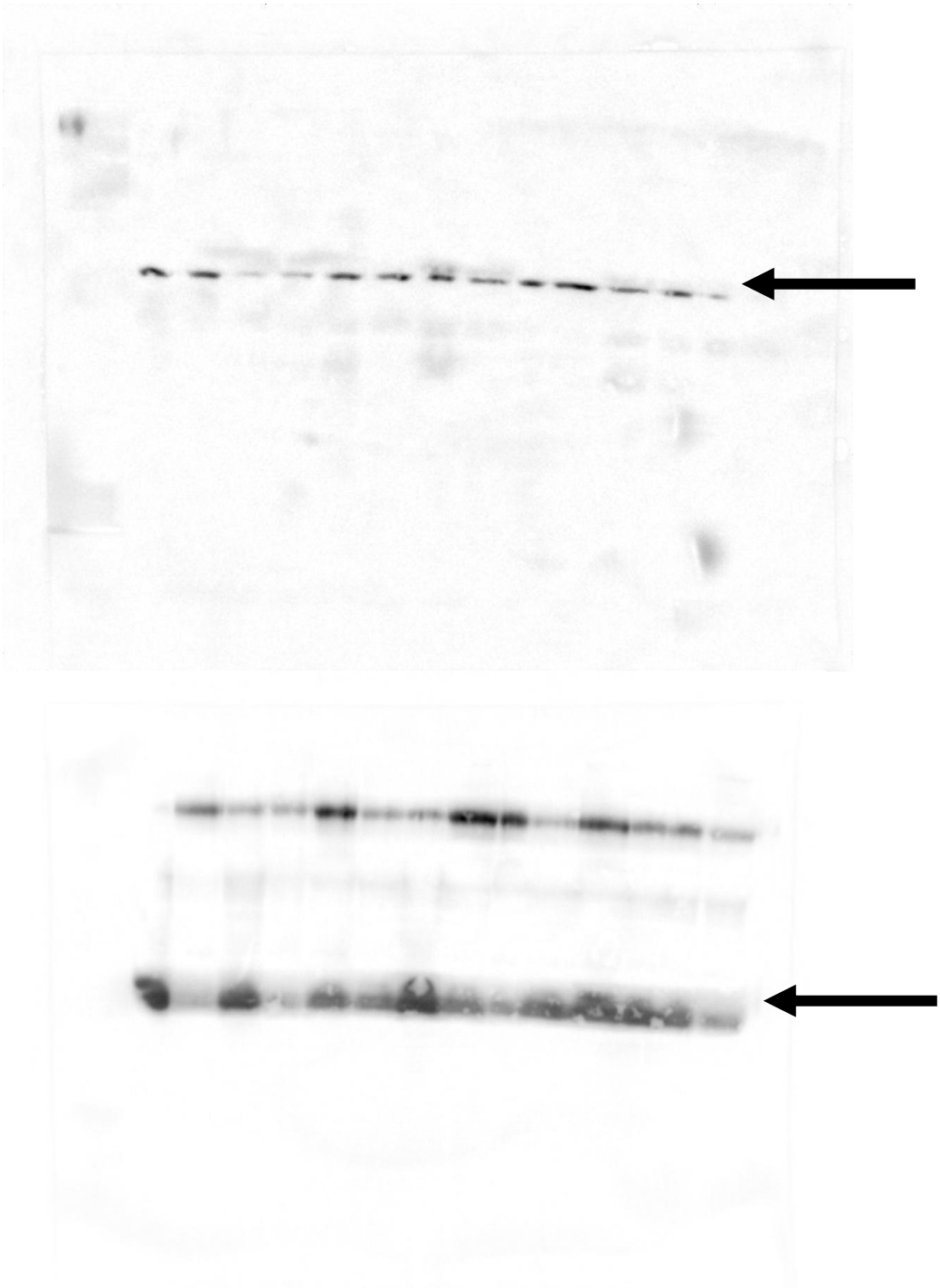

Figure C.1 - Representative Western blots of PDK2 (top) and actin (bottom). Blots are plotted as WT, KO, WT, KO

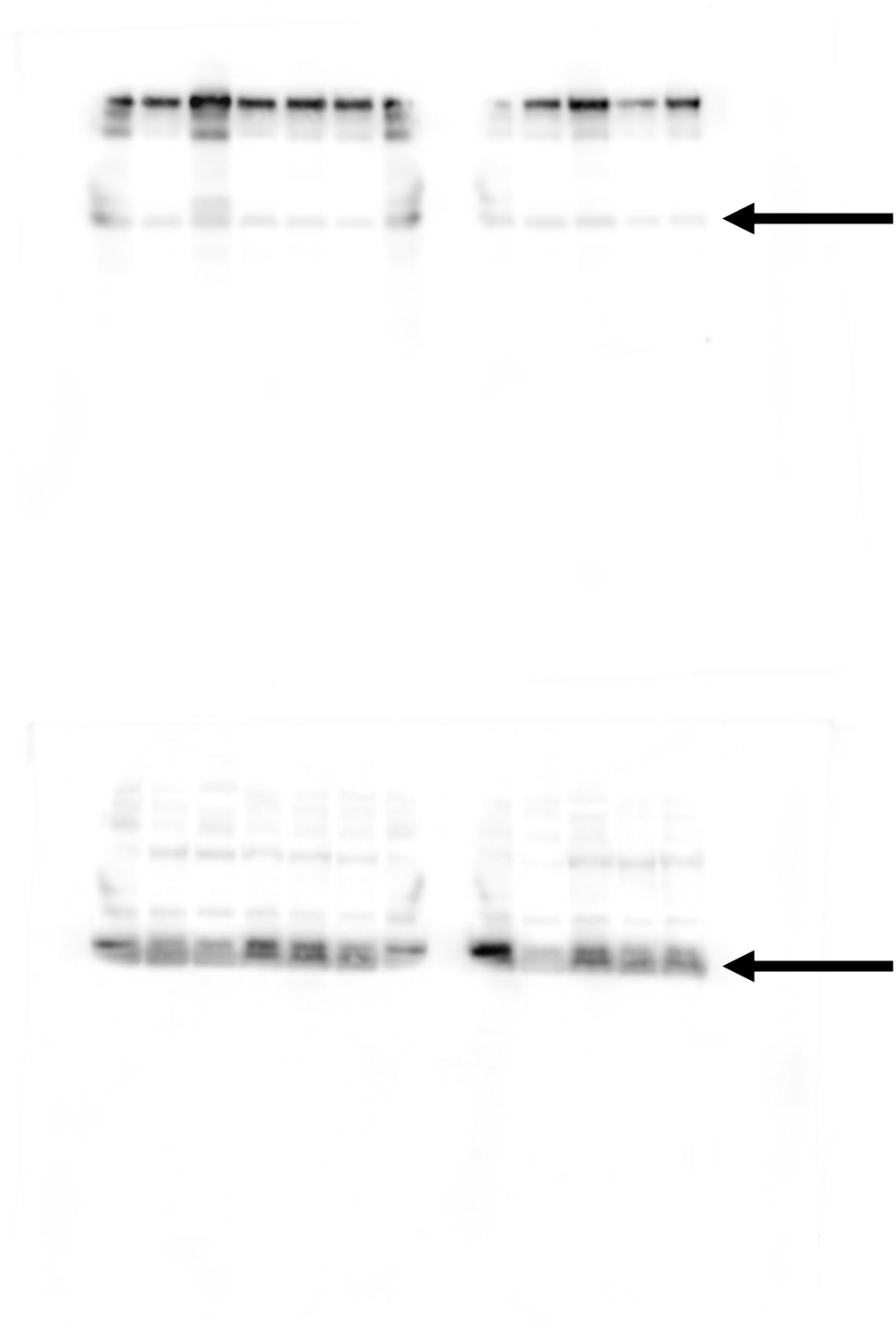

Figure C.2 – Representative Western blots of PDP1 (top) and actin (bottom). Blots are plotted as WT, KO, WT, KO